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The Effect of TMPRSS6 A736V Polymorphism on Hepcidin Level and Iron Metabolism in Chronic Hemodialysis Patients in Jenin City-Palestine

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Thesis Approval

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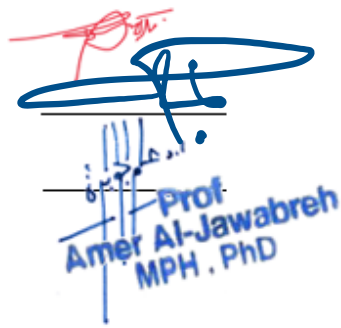

Declaration

I certify that this dissertation is primarily my own work and has not been submitted for consideration for any other degree at Arab American University or any other institution, with the exception of instances in which the contributions of others are specifically acknowledged.

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Dedication

I dedicate this thesis to my parents, in deepest appreciation of their unwavering love, guidance, and endless support throughout my life. Your values, sacrifices, and constant encouragement have shaped who I am today and have been the foundation of every achievement I have reached. To my brothers, thank you for standing by me in every circumstance and for being a constant source of strength, comfort, and motivation. Your presence and support have meant more to me than words can express.

I also dedicate this work to my supportive friends, whose encouragement, kindness, and belief in me carried me through difficult moments. Your words, patience, and companionship gave me the strength to continue when the journey felt overwhelming.

This thesis is a reflection of the love, support, and faith of those who surrounded me, combined with my own perseverance and dedication. To all of you who believed in me and walked beside me on this path, this work is lovingly dedicated.

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I also extend my heartfelt appreciation to all those who offered assistance and encouragement during this journey. Special thanks go to my colleagues, whose support, cooperation, and positive spirit made this experience both smoother and more enjoyable.

Abstract

Background: Hepcidin is the principal regulator of systemic iron homeostasis. It controls dietary iron absorption and iron release from macrophages by binding to and inducing the degradation of ferroportin, the only known cellular iron exporter. Hepcidin expression is tightly regulated by body iron stores, inflammation (particularly IL-6 signaling), erythropoietic activity, and genetic factors. TMPRSS6, which encodes the serine protease matriptase-2, acts as a negative regulator of hepcidin by inhibiting the BMP/SMAD signaling pathway. In chronic hemodialysis (CHD) patients, hepcidin levels are frequently elevated due to reduced renal clearance and persistent inflammation, leading to functional iron deficiency, anemia, and increased requirements for erythropoiesis-stimulating agents and intravenous iron therapy. The TMPRSS6 rs855791 (A736V) polymorphism modulates matriptase-2 activity; the T allele has been associated with higher hepcidin expression and reduced iron availability.

Objectives: To assess the impact of TMPRSS6 p.A736V on serum hepcidin levels and erythropoiesis-related parameters in Palestinian CHD patients, aiming to improve anemia management and optimize iron and drug therapy.

Methods: Hematological, biochemical, and inflammatory parameters were obtained from medical records and questionnaires. Blood samples were collected for serum hepcidin measurement (ELISA) and TMPRSS6 rs855791 genotyping using allele-specific PCR.

Results: Fifty CHD patients and 50 healthy controls were studied. CHD patients had significantly higher hepcidin (median 525 vs. 78.5 ng/ml, $p < 0.001$). The CT genotype was most common (66%), followed by CC (23%) and TT (11%), with a stepwise increase in hepcidin from CC to CT to TT. Hepcidin correlated positively with ferritin (rho = 0.302, $p = 0.033$) and creatinine (rho = 0.566, $p < 0.001$), but not with hemoglobin, MCV, RDW, or transferrin. Sex

and smoking had no effect. Diabetes and hypertension were the main causes of end-stage renal disease.

Conclusion: Hepcidin is elevated in CHD patients in a TMPRSS6 genotype-dependent manner. These findings underscore the need to consider TMPRSS6 variation when managing anemia and tailoring iron and erythropoiesis-stimulating therapy in CHD patients.

Keywords: Chronic hemodialysis, Hepcidin, TMPRSS6, A736V, Iron metabolism, Anemia, Ferritin, Genetic variation.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Definitions of Abbreviations

Abbreviation	Full term
CKD	Chronic Kidney Disease
ESKD	End-Stage Kidney Disease
ESRD	End-Stage Renal Disease
CHD	Chronic Hemodialysis
HD	Hemodialysis
RRT	Renal Replacement Therapy
AKI	Acute Kidney Injury
eGFR	Estimated Glomerular Filtration Rate
GFR	Glomerular Filtration Rate
KDIGO	Kidney Disease Improving Global Outcomes
NKF-KDOQI	National Kidney Foundation – Kidney Disease Outcomes Quality Initiative
T2DM	Type 2 Diabetes Mellitus
WHO	World Health Organization
Hb	Hemoglobin
RBC	Red Blood Cells
EPO	Erythropoietin
ESA	Erythropoiesis-Stimulating Agent
TS	Transferrin Saturation
TIBC	Total Iron-Binding Capacity
Fe	Iron
Fe^{2+}	Ferrous Iron
Fe^{3+}	Ferric Iron
NTBI	Non-Transferrin-Bound Iron
LPI	Labile Plasma Iron
Tf	Transferrin
TfR1	Transferrin Receptor 1
TfR2	Transferrin Receptor 2
DMT1	Divalent Metal Transporter 1
FPN (FPN1)	Ferroportin
DCYTB	Duodenal Cytochrome B
STEAP3	Six-Transmembrane Epithelial Antigen of the Prostate 3
Fe–S	Iron–Sulfur Clusters
H_2O_2	Hydrogen Peroxide

Abbreviation	Full term
IRPs	Iron Regulatory Proteins
IREs	Iron-Responsive Elements
UTR	Untranslated Region
HFE	Hereditary Hemochromatosis Protein
HH	Hereditary Hemochromatosis
IRIDA	Iron-Refractory Iron Deficiency Anemia
TMPRSS6	Transmembrane Protease Serine 6 (Matriptase-2)
rs855791	Single-Nucleotide Polymorphism ID
p.A736V	Alanine to Valine Substitution at Position 736
MT2736A/V	Matriptase-2 Amino Acid Variants
BMP	Bone Morphogenetic Protein
BMP6	Bone Morphogenetic Protein-6
BMPR	Bone Morphogenetic Protein Receptor
HJV	Hemojuvelin
HAMP	Hepcidin Antimicrobial Peptide Gene
TGF-β	Transforming Growth Factor-beta
GPI	Glycosylphosphatidylinositol
SMAD	Mothers Against Decapentaplegic Proteins
pSMADs	Phosphorylated SMAD Proteins
IL-6	Interleukin-6
JAK1/2	Janus Kinase ½
STAT3	Signal Transducer and Activator of Transcription 3
ERFE	Erythroferrone
CUB	Complement C1r/C1s, Uegf, Bmp1 Domain
LDLR	Low-Density Lipoprotein Receptor
APR	Acute Phase Reactant
CRP	C-Reactive

Chapter One: Introduction

1. Introduction:

1.1. Background

Chronic kidney disease (CKD) is complex condition, which characterized mainly by progressive renal dysfunction, that can lead to end-stage kidney disease (ESKD) and cardiovascular complications. The various metabolic and systemic disorders associated with CKD further accelerate its progression and increase the risk of morbidity related to cardiovascular.

Although CKD is highly prevalent and associated with significant clinical burdens, the public knowledge about the disease remains low, and the studies showed that only about 6% of the population and 10% of individuals at high risk are aware of CKD statuses (Ene-Iordache et al., 2016). This is mostly because CKD remains silent until its late stages. Unfortunately, identifying the disease late decrease the chances to prevent serious outcomes.

Recently, CKD has no direct cure, rather, there are treatments that mainly reducing cardiovascular risk and slowing disease progression. Even with treatment options, patients continue to face a continues risk of further kidney decline and other outcomes. Many studies focused on cardiovascular disease, in CKD patients, and reported that it may associated with aging, hypertension, Type 2 Diabetes Mellitus (T2DM) and dyslipidemia (Evans et al., 2022).

In order to manage CKD and give better care to those patients, National Kidney Foundation Kidney Disease Outcomes Quality Initiative and the international guideline group Kidney Disease Improving Global Outcomes (KDIGO) (Levey et al., 2005), developed a classification to CKD, according to estimated glomerular filtration rate (eGFR) and albuminuria. There are six eGFR categories, in which the severity of kidney damage, increases with decreasing eGFR measurements, ESRD considered the last one of them, it is diagnosed when function of the kidney is no longer adequate for survival without transplantation or dialysis. Typically, kidney transplantation leads to better patient outcomes, but most patients are treated with dialysis (Wouk, n.d.).

Patients with ESRD frequently undergoing chronic hemodialysis (CHD), and usually suffering from anemia, related to insufficient erythropoietin (EPO) production, absolute iron deficiency, because of recurrent blood loss, or functional iron deficiency because of high hepcidin, and the presence of chronic inflammation (Alves et al., 2015).

The standard therapy includes erythropoiesis-stimulating agents, and intravenous iron supplementation to the patients. However, keeping the levels of hemoglobin (Hb) on target ranges is often challenging, due to variable responses and various side effects (Canavesi & Valenti, 2011).

One of the most altered parameters in ESRD, is iron metabolism parameters, commonly appear as low transferrin saturation (TS) and elevated serum ferritin. These defects have been associated with increased levels of hepcidin, which is a hepatic hormone that serves as the key regulator of systemic iron balance (Pelusi et al., 2013a, 2013b).

Elevated hepcidin levels restricts intestinal iron absorption and inhibits its recycling from macrophages, by binding to and degrading ferroportin, the known exporter of iron. Therefore, iron becomes trapped within cells, decreasing its availability for erythropoiesis (Canavesi & Valenti, 2011).

In CHD patients, hepcidin upregulation has been associated with impaired renal clearance, chronic inflammation, and iron accumulation due to repeated supplementation, whereas conditions such as anemia, hypoxia, and EPO signaling suppress its expression (Tsuchiya & Nitta, 2013).

The transcriptional regulation of hepcidin involves a complex proteins network, including the hereditary hemochromatosis protein (HFE), and type II transmembrane protease serine 6, which also known as matriptase-2 (TMPRSS6). Matriptase-2 downregulates hepcidin by cleaving hemojuvelin – (which acts as a co-receptor in transmembrane-status on BMPs bone morphogenetic protein (BMP) signaling to stimulate hepcidin production), thus subsequently regulates the iron levels in the body (Pelusi et al., 2013b).

Rare mutations in TMPRSS6 leading to iron-refractory iron-deficiency anemia (IRIDA), because of the high hepcidin levels (Valenti et al., 2012). In contrast, the common

variant rs855791 (p.A736V) had recognized as major determinant for iron status in general population, and that carriers of the A736V allele usually have lower serum iron, higher hepcidin concentrations, and reduced Hb, suggesting lower efficient suppression for hepcidin transcription (Canavesi & Valenti, 2011). This polymorphism has also implicated in conditions of iron overload such as hereditary hemochromatosis (HH) and nonalcoholic fatty liver disease.

The study of A736V polymorphism on gene TMPRSS6 focuses on its effect on hepcidin regulation and iron metabolism among CHD patients in Jenin, Palestine. Given that hepcidin plays an important role in iron homeostasis, genetic variations influencing its expression may contribute to treatment resistance and the difficulty of managing anemia in this population.

Although previous research has addressed this polymorphism in other populations, no study has explored its relevance in Palestinian patients yet.

So, this work seeks to address this gap, offering new informations that may improve prognosis and inform more effective therapeutic strategies to improve local clinical settings.

1.2. Significance of the Study

In 2020, World Health Organization (WHO) ranked CKD as the 10th leading cause of death. It is expected to be the fifth leading cause of death by 2040 (Valenti et al., 2012) and this is a serious matter to be concerned. Especially in the West Bank, the number of CHD patients has increased four folds since 2009, and chronic kidney failure ranked as the ninth leading cause of death in Palestine (Palestinian Ministry of Health, 2021).

No much data were found about the total number of Hemodialysis patient number in Palestinian hospitals, but according to the Palestinian Ministry of Health in 2020, 1,573 patients were undergoing regular hemodialysis sessions (Palestinian News & Information Agency – WAFA, 2021), while it increased to 1,601 in 2022, and to 1,731 patients in 2023 (Palestinian News & Information Agency – WAFA, 2024), which consider a high increase in such period of time, as shown in Figure 1.1.

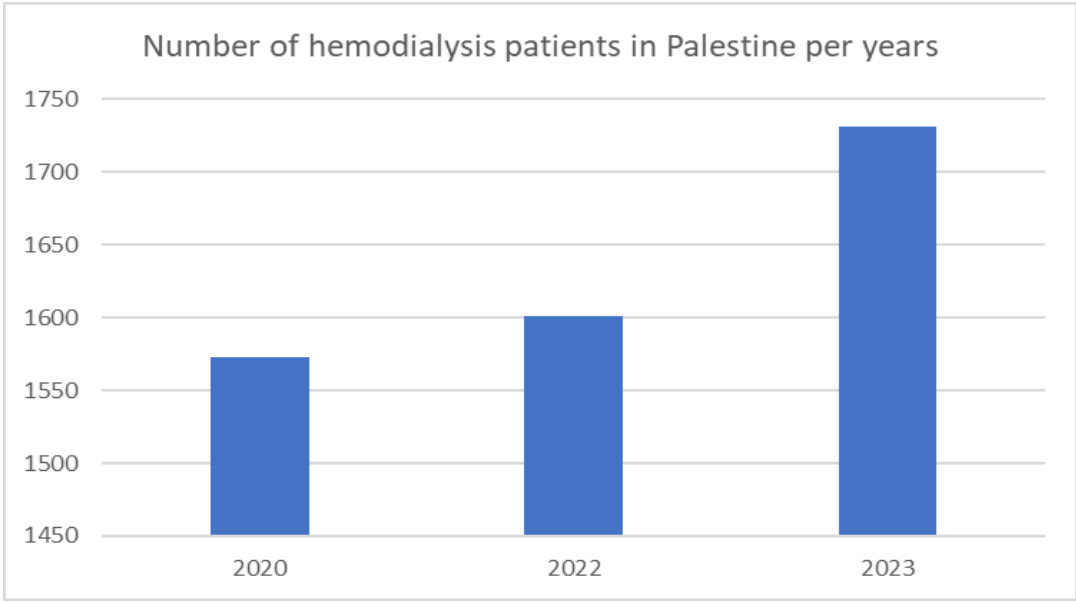

Figure 1.1 Number of hemodialysis patients in Palestine per years.

In order to predict the results of hepcidin levels to improve therapy, anemia management, and the appropriate medicine dosages, to prevent the danger of diseases connected to severe iron supplementation, and enhancing patient outcomes, the study aims to determine the effect of TMPRSS6 p.A736V polymorphism on hepcidin levels and erythropoiesis parameters, such as Hb, serum EPO, and RBC count, in Palestinian CHD patients. This will add to existing knowledge states that the p.A736V variant less effectively inhibits hepcidin transcription so greater hepcidin levels, linked to lower blood iron, and decreased Hb in the healthy population (Pelusi et al., 2013a).

1.3. Statement of the Problem

The increase in mortality rate among CHD patients ranging from 7.4% and 8.0% (J. Liu et al., 2023) has been a major problem. Different causes of high mortality rate have been reported, including side effects of high iron supplementation doses, such as sepsis (Portolés et al., 2021). Also, low EPO levels in some patients, which related to cardiovascular diseases (Won et al., 2014).

One important mechanism is to extract the DNA, and study the effect of the polymorphism by Allele Specific-Polymerase Chain Reaction (AS-PCR), as will be described in methodology chapter. Therefore, it is essential to study the effect of TMPRSS6

rs855791 C > T polymorphism, which regulates iron metabolism by altering hepcidin levels. Understanding its role could change the treatment plan to avoid decrease iron, EPO, level, thus decreasing death rate.

1.4. Objectives of the Study

The purpose of this study was to determine if serum levels of hepcidin, the hormone that controls iron metabolism, and erythropoiesis, are influenced by the A736V TMPRSS6 polymorphism, a significant genetic driver of iron metabolism in healthy persons, in CHD in Jenin city from November 2024, to August 2025.

The specific objectives are:

1. To determine the prevalence of the A736V TMPRSS6 polymorphism in CHD and healthy population in Jenin city by AS-PCR.
2. To determine the level of the hormone hepcidin in serum samples by ELISA.
3. To determine the effect of A736V TMPRSS6 polymorphism hepcidin level and iron metabolism.

1.5. Research Questions

1. Does the TMPRSS6 A736V variant influence the serum levels of hepcidin hormone among CHD patients?
2. Is there an association between the TMPRSS6 A736V polymorphism and iron metabolism/erythropoiesis in CHD patients?
3. Does the presence of the A736V polymorphism affect the iron supplementation doses required for CHD patients?

1.6. Hypotheses of the Study

1.6.1. Null Hypotheses (H0)

1. The TMPRSS6 A736V variant does not influence serum levels of hepcidin hormone in CHD patients.
2. The TMPRSS6 A736V variant does not affect iron metabolism or erythropoiesis in CHD patients.
3. The TMPRSS6 A736V variant does not influence the iron supplementation doses required among CHD patients.

1.6.2. Alternative Hypotheses (H1)

1. The TMPRSS6 A736V variant influences serum levels of hepcidin hormone in CHD patients.
2. The TMPRSS6 A736V variant affects iron metabolism and erythropoiesis in CHD patients.
3. The TMPRSS6 A736V variant influences the iron supplementation doses required among CHD patients.

1.7. Conceptual and Operational Definitions

1.7.1. Conceptual Definitions

- A736V polymorphism (TMPRSS6 gene): A genetic variation in the TMPRSS6 gene, in which alanine is replaced by valine at position 736. This variation may influence iron metabolism by modulating the production or activity of hepcidin hormone (Nai et al., 2011).
- Hepcidin hormone: A peptide hormone synthesized by the liver that regulates systemic iron homeostasis by controlling intestinal iron absorption and release of iron from macrophages (Tsuchiya & Nitta, 2013).
- Iron metabolism: The physiological processes involved in the absorption, transport, storage, and utilization of iron in the human body (Roemhild et al., 2021).
- CHD patients: Individuals with end-stage renal disease undergoing regular hemodialysis to maintain fluid and electrolyte balance.

1.7.2. Operational Definitions

- A736V polymorphism (TMPRSS6 gene): Determined using AS-PCR performed on DNA extracted from patients' blood samples (Valenti et al., 2012).
- Hepcidin levels: Measured in serum using a validated ELISA kit according to manufacturer instructions (Valenti et al., 2012).
- Iron metabolism parameters: Extracted from patient medical records, including serum ferritin (ng/mL), serum iron (µg/dL), total iron-binding capacity (µg/dL) (TIBC), and transferrin level (mg/dL).
- CHD patients: Adult patients attending dialysis units in Jenin city, who meet the inclusion and exclusion criteria of the study as will be discussed in methodology

part, with blood samples collected in the morning before dialysis, at least three days after the last EPO dose, and one week after the last intravenous iron administration to avoid confounding effects on iron and hepcidin levels.

Chapter Two: Literature Review

2- Literature Review:

Iron (Fe), one of the most important elements, for many biological functions in the body, and one of the most abundant elements in the earth, its unique capacity to donate and receive electrons during redox reactions makes it indispensable for many essential biological functions (Dev & Babitt, 2017). One of iron functions, is incorporating into hemoglobin inside RBCs. When erythrocytes aged, macrophages broke them down in the spleen, liver, and bone marrow, allowing their iron to be recycled. enterocytes absorbed dietary iron by Divalent Metal Transporter 1 (DMT1), then released into the bloodstream via ferroportin (FPN), where it binds to transferrin and is delivered to tissues through Transferrin Receptor 1 (TfR1) (Vogt et al., 2021b).

Hemoglobin contains most of the active iron, while the liver stores the largest reserve in ferritin. Usually, when iron levels increase, hepatocytes produce hepcidin, which binds to FPN, triggers its degradation, leading to limit iron release in the circulation. These steps maintain iron balance at both cellular and systemic levels (Vogt et al., 2021b). The literature review below summarizes the key mechanisms involved in cellular and systemic iron regulation. Iron is considered one of the key metals needed for life, for both simple single-celled organisms, and complex multicellular species, like Humans. It participates in numerous cellular processes, including DNA synthesis and repair, mitochondrial respiration, cell growth, and programmed cell death. As Well as in immune defense mechanisms and cellular signaling pathways (Vogt et al., 2021b).

In addition to many cellular functions, it has an important role in the formation of one of the essential components of hemoglobin (Hb), the heme, that enables RBCs to carry and deliver oxygen within the body. iron can shift between oxidation states, in human body, as ferrous (Fe^{2+}) or as ferric (Fe^{3+}) (Sukhbaatar & Weichhart, 2018). Although iron is vital in maintaining normal physiology, under certain conditions, it also can be harmful. particularly in presence of hydrogen peroxide (H_2O_2). Fe^{2+} can interact with hydrogen peroxide to produce highly reactive hydroxyl radicals, while being converted to Fe^{3+}. These radicals,

capable of inflicting oxidative stress, damaging lipids and cellular structures, and may lead to tissue injury and programmed cell death (Jomova & Valko, 2011).

Because excess iron could be toxic, the body must carefully regulate its levels, to prevent iron overload cases. On the other hand, the long-term iron deficiency reduces the iron that is available for the bone marrow, leading to restricted RBCs production, known as iron-restricted erythropoiesis, which can cause moderate to severe anemia. Even when anemia isn't present, continues iron deficiency has been linked to fatigue, impaired immune function, and worse outcomes, in conditions as heart failure (Cappellini et al., 2020).

Iron Flow in the Human Body

Most of the iron in the human body, about 80%, is found in hemoglobin inside red blood cells. The remaining iron is stored in macrophages and liver cells or used in other heme proteins and iron sulfur (Fe-S) clusters. Most of this iron supports erythropoiesis process, that producing new red blood cells that transport oxygen throughout the body (Cronin et al., 2019). Each red blood cell carries roughly 280 million hemoglobin molecules, which means it contains more than a billion iron atoms. Hemoglobin's main function to bind oxygen and deliver it to tissues. because the amount of circulating iron is small compared with the body's daily needs, iron must be constantly recycled from aging red blood cells to sustain erythropoiesis and meet the body's overall iron needs (Cassat & Skaar, 2013).

Only small amount of iron actually comes from what we absorb through our diet, about 1–2 mg per day. Usually, Dietary iron found in two forms, the heme and non-heme iron. Heme iron, that come mainly from hemoglobin and myoglobin in meat and poultry, while non-heme iron, is found in plant-based foods like cereals and vegetables. Because body recycle iron so efficiently, absorbing just 1–2 mg daily is usually enough to keep overall balance, and replace iron lost through shedding cells. Before non-heme dietary iron, –which is mostly Fe^{3+} form, can be absorbed, it must first be converted into Fe^{2+} in the intestine (Fleming & Ponka, 2012a).

Fe^{3+} is converted to its Fe^{2+} form by ferric reductase enzyme, duodenal cytochrome B (DCYTB), located on the apical surface of enterocytes facing the intestinal lumen. when reduction done, Fe^{2+} enters the enterocytes through the divalent metal transporter 1 (DMT1), which is the primary transporter responsible for iron absorption in the duodenum and upper ileum. After iron entering to cell, it can be used immediately for the enterocyte's metabolic

needs, stored in ferritin, or exported across the basolateral membrane for delivery to the rest of the body (Li et al., 2020). After being absorbed by enterocytes, Fe^{2+} is exported into the bloodstream through FPN1, the body's only known iron-exporting protein. When it exits the cell, Fe^{2+} is oxidized back to Fe^{3+}, by either hephaestin or ceruloplasmin, allowing it to bind to transferrin (Tf), the main iron-transport protein in the plasma, for distribution throughout the body (Nairz et al., 2017) Figure 2.1.

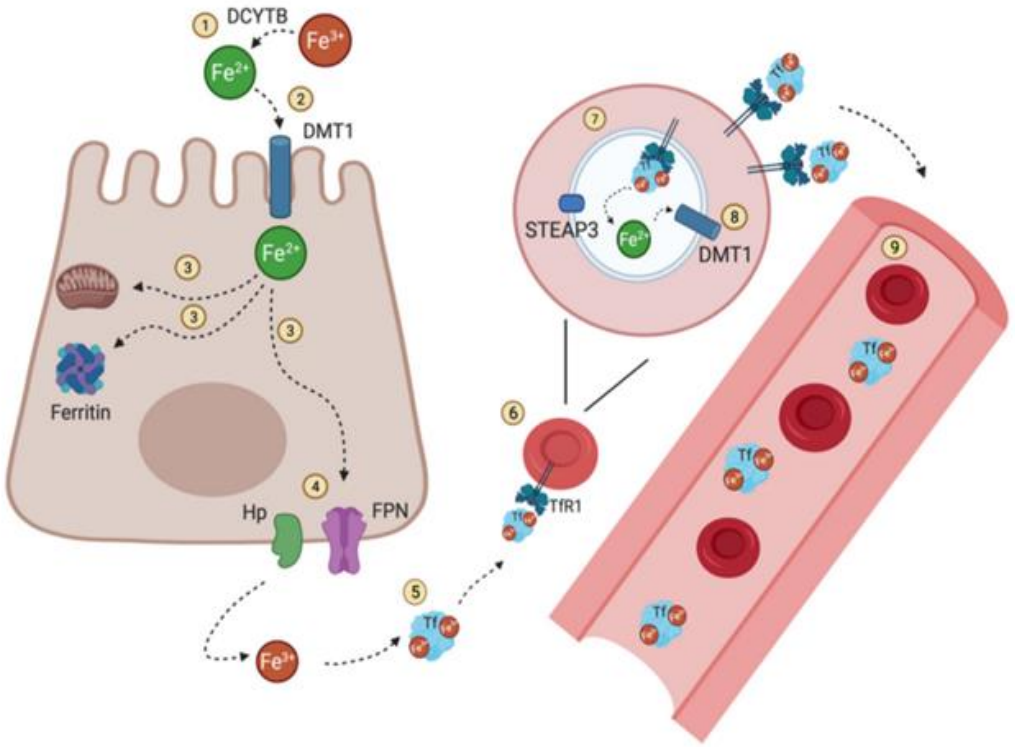

Figure 2.1 Iron distribution and circulation, (Vogt et al., 2021b).

Transferrin (Tf) has two high-affinity binding sites for Fe^{3+} and serves as the main glycoprotein that responsible of transport ferric iron, while keeping it in a non-reactive form. Under normal conditions, usually all circulating iron is bound to transferrin. while, in certain pathological situations where transferrin's binding capacity is exceeded, non-transferrin-bound iron (NTBI) start to appear, and this form of iron can be taken up by the liver and may lead to toxic effects (Anderson, 2005).

A redox-active fraction of NTBI, is known as labile plasma iron (LPI), is not available for erythropoiesis. Instead, LPI can enter non-hematopoietic cells, causing iron accumulation in tissues and potentially leading to free radical–mediated damage. Most of iron that

circulates in the blood is carried by transferrin. In the bone marrow, erythroid precursor cells rely mostly on transferrin-bound iron, which they take up through transferrin receptor 1 (TfR1), which expressed at high levels on their surface. In contrast, hepatocytes and other non-erythroid cells can also utilize NTBI and additional iron sources because they express a broader range of iron transporters (Waldvogel-Abramowski et al., 2014).

Circulating iron that reaches erythroid precursors and other cells is taken up by receptor-mediated endocytosis into clathrin-coated pits. TfR1 stabilized by disulfide bonds and have high affinity for transferrin carrying two Fe^{3+} ions (diferric Tf). When diferric Tf binds to TfR1, the complex is internalized through clathrin-dependent endocytosis. The interaction is pH-dependent, as the endosome acidifies, the iron bound to transferrin is released (Fleming & Ponka, 2012a). After its release in the endosome, iron is reduced to Fe^{2+} by STEAP3 then transported into the cytosol through DMT1, while TfR1 is recycled back to the cell surface. once enter the cytosol, iron go to labile pool for iron, where it could be used immediately, as in case of heme synthesis, or stored in cytosolic ferritin (Fleming & Ponka, 2012b).

Ferritin, is 24-subunit protein made up of heavy (H) and light (L) chains, these subunits form a hollow in its structure, capable of storing as many as 4,500 iron atoms, making ferritin the body's primary intracellular iron storage protein. Within this structure, iron is stored as Fe^{3+} in safely sequestered form, to prevents formation of harmful reactive oxygen species. Ferritin-bound iron is the main storage form of iron, in macrophages and liver (Vogt et al., 2021c) as in Figure 2.2.

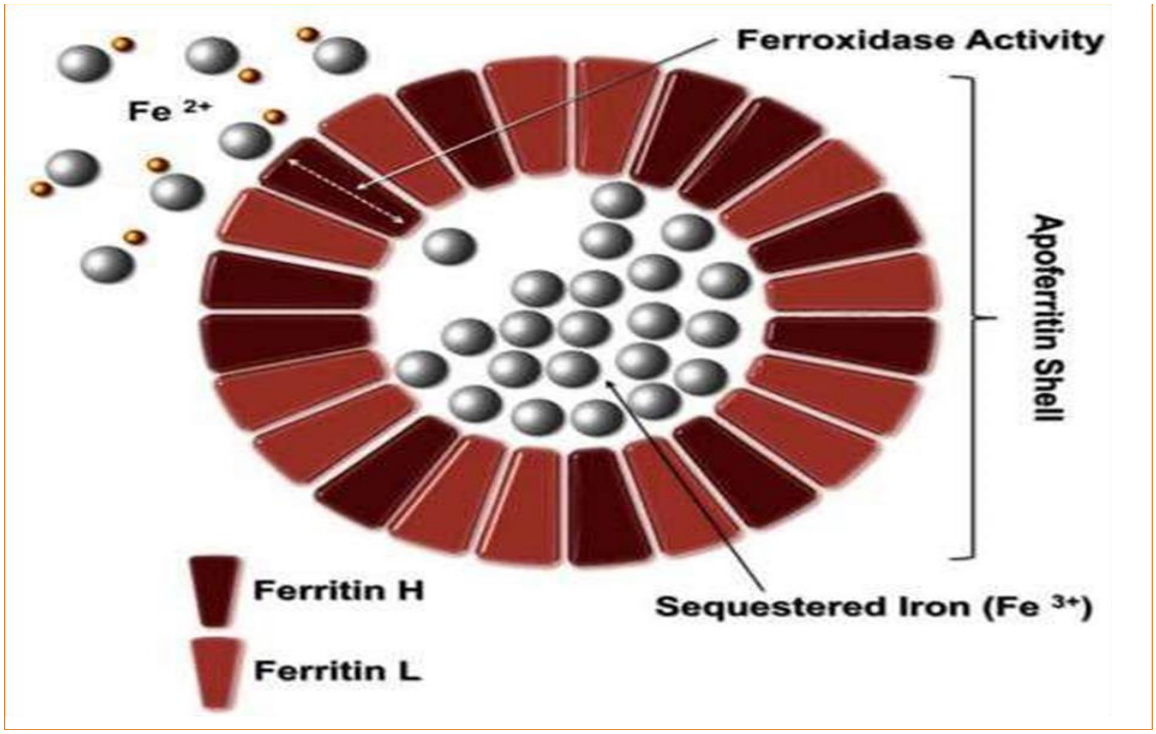

Figure 2.2 Ferritin shape (Knovich et al., 2009).

Liver the Central Organ in Iron Homeostasis

The liver, particularly its hepatocytes, play central role in iron metabolism. Hepatocytes make up 80% of the liver's mass and relate significantly to the body's iron regulation. The liver hepatocytes produce large amounts of Ferritin, which allows the liver to serve as a main reservoir for absorbed iron. The liver is also responsible for producing most of the transferrin (Daher & Karim, 2017). Liver hepatocytes also serve as regulators of iron homeostasis by producing and releasing the 25–amino-acid peptide hormone hepcidin. It is secreted into bloodstream, hepcidin function is to inhibit iron release from various cells, including duodenal enterocytes, macrophages, hepatocytes, and Kupffer cells, by binding to FPN exporter, triggering FPN's ubiquitination, internalization, and degradation, while also directly blocking its channel. This effectively prevents iron from being exported from cells into the plasma (Li et al., 2020).

Hepcidin production, transcriptionally regulated, in response to levels of serum iron. When serum iron levels increase, hepcidin expression increase, and acts as negative feedback mechanism, to block iron transport into the plasma via ferroportin. preventing potentially toxic iron accumulation in the body. Conversely, when plasma iron levels decrease,

transferrin saturation decreases, leading to reduced hepcidin production (Daher & Karim, 2017).

As a result, concentrations of iron in biological fluids are regulated to ensure sufficient intracellular and extracellular iron, and avoiding toxicity. This regulation is critical, because any disruption in iron or content can negatively impact essential physiological processes (Li et al., 2020).

Iron Regulation

Cellular Regulation of Iron—The Iron Regulating Proteins (IRPs)

Iron metabolism is controlled at both systemic and cellular levels. the key protein responsible of iron transport is TfR1. Diferric Tf binds to TfR1 and is internalized via clathrin-mediated endocytosis. Acidification of the endosome induces conformational change in both transferrinm and TfR1, causing iron to dissociate from transferrin. The Tf–TfR1 complex is then recycled back to the plasma membrane ("Contents, Ed. Board + Forthc. Articles," 2005). A related protein, transferrin receptor 2 (TfR2), is expressed widely in hepatocytes. At the cellular level, the expression of iron-related proteins, as ferritin, and transferrin receptors, is regulated by iron-sensing proteins, known as iron regulatory proteins (IRPs) or IRE-binding proteins, which interact with iron-responsive elements (IREs) to maintain iron homeostasis by regulating gene expression post transcriptionally (Chen & Paw, 2012).

Sensing and Regulating Intracellular Iron by IRP1 and IRP2

The intracellular iron pool, regulated by interaction of IRP1 and IRP2 with IREs. These RNA-binding proteins control the translation of key proteins that are involved in iron metabolism. IREs can be located in either the 5′ or 3′ untranslated regions (UTRs) of target mRNAs. When IRP binds to IRE in the 5′ UTR, translation of the mRNA inhibited. In contrast, binding of IRP to IRE in the 3′ UTR stabilizes the mRNA, resulting in increased translation, by protects the transcript from endonucleolytic degradation (Vogt et al., 2021b) Figure 2.3.

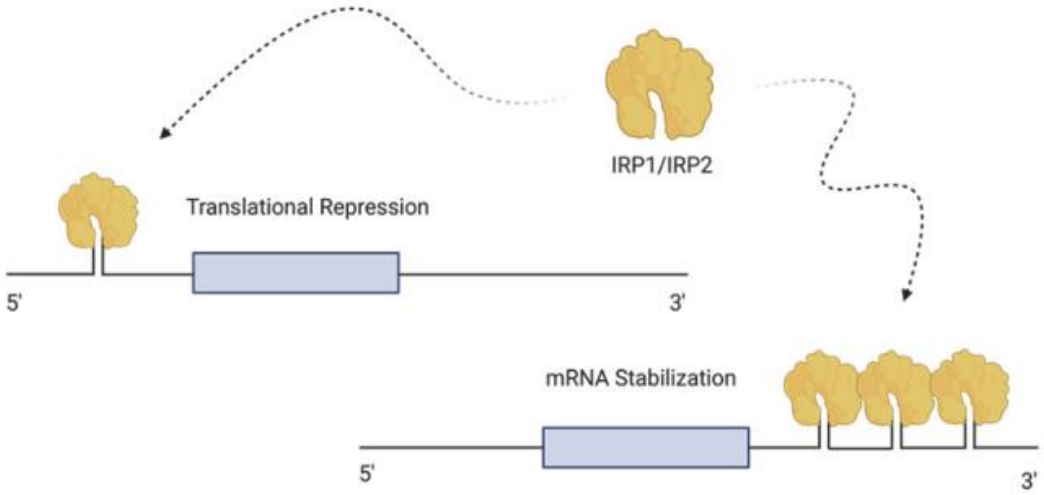

Figure 2.3 Cellular iron regulation, (Vogt et al., 2021b).

Systemic Regulation of Iron—The Hepcidin–Ferroportin Axis

At the systemic level, iron homeostasis mainly controlled by the hepcidin–ferroportin axis. Hepcidin acts as a negative regulator of iron flow, While FPN plays a role in controlling iron release from enterocytes, hepatocytes, and macrophages. Structurally, FPN consists of 12 transmembrane helices arranged into two lobes. These lobes can switch between two conformations. In the active state, the central cavity of FPN faces the intracellular space, making it inaccessible from the extracellular side. In the alternate state, FPN's central cavity faces the extracellular space, making it inaccessible from the inside of the cell (Cronin et al., 2019).

Under normal conditions, iron is released from major iron stores through FPN. To maintain proper plasma and tissue iron levels, FPN expression is tightly controlled post-translationally by circulating hepcidin. Disruption of its regulation can lead to iron-related disorders. A deficiency of hepcidin leads to iron overload in hepatocytes, as seen in conditions like hereditary hemochromatosis. Conversely, excessive hepcidin production is linked to iron-restricted anemia (Sangkhae & Nemeth, 2017).

Hepcidin, primarily regulated at transcriptional level, and mainly it is produced by liver hepatocytes. After synthesis, it undergoes proteolytic process, to generate the active peptide, which released into the bloodstream, where it can bind to, and inhibit FPN activity (Steinbicker & Muckenthaler, 2013).

In addition to hepatocytes, monocytes, macrophages, the kidney can produce hepcidin, though to a lesser extent. When hepcidin bind to FPN, it triggers internalization, and lysosomal degradation of the iron exporter. This blocks iron export, causing iron to be retained, within FPN-expressing cells (Steinbicker & Muckenthaler, 2013) Figure 2.4.

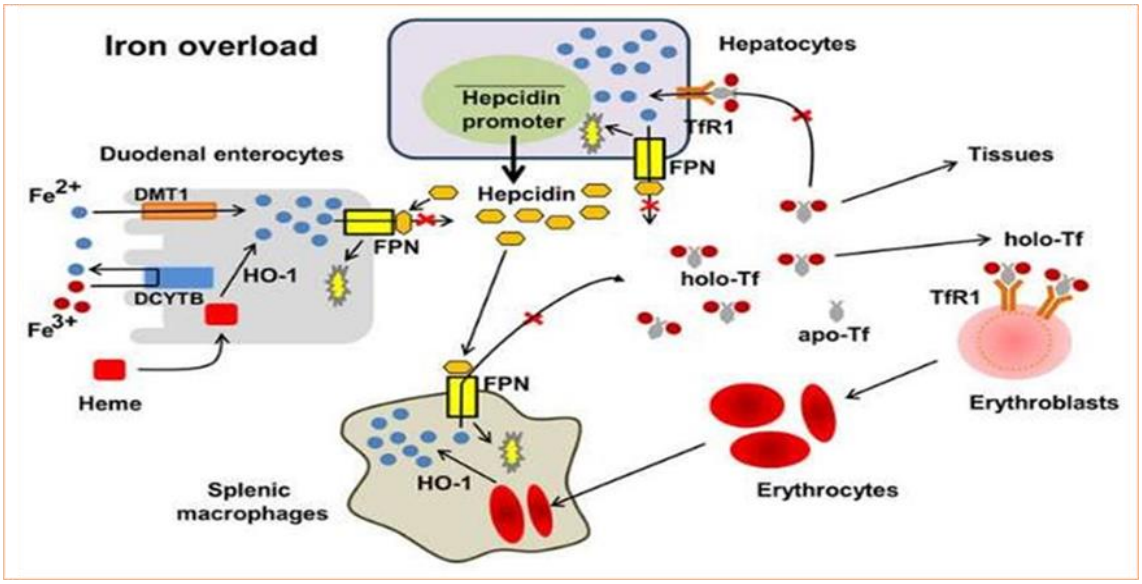

Figure 2.4 Regulation of hepcidin according to body iron levels (Ueda & Takasawa, 2018a).

During iron overload, hepcidin levels rise, to prevent iron export through FPN. So, when circulating iron levels increase and cellular uptake is high, iron remains stored in cells, rather than accumulating in the extracellular space. But Hepcidin production is suppressed during anemia or ineffective erythropoiesis but is stimulated in conditions of high iron load or inflammation (Kautz et al., 2014).

Regulation of Hepcidin through the Bone Morphogenetic Protein

Hepcidin expression is mainly controlled at the transcriptional level through a feedback loop, that allow hepatocytes to sense body's circulating iron and its amount. This process is complex, and depends on several membrane-associated proteins, including the hereditary hemochromatosis protein (HFE), transferrin receptor 2 (TfR2), and hemojuvelin (HJV). Together, these proteins help in hepcidin production, by modulating signals through bone morphogenetic protein 6 (BMP-6) (Ueda & Takasawa, 2018b).

BMP-6, is extracellular signaling molecule, that belong to transforming growth factor-β (TGF-β) family, it is produced by hepatocytes and plays a main role in activating

hepcidin expression. Increased intracellular iron levels in the liver stimulate the expression of BMP6, which in turn binds to its receptor (BMPR) together with hemojuvelin (HJV), a glycosilphosphatidylinositol (GPI)-anchored protein (Papanikolaou et al., 2004), which act as a co-receptor, it activates intracellular signaling pathways in hepatocytes through son of mothers decapentaplegic (SMAD) proteins (Cappellini et al., 2020). This pathway of signals leading to the phosphorylation of SMAD1, SMAD5, and SMAD8 (pSMADs), which form complexes with SMAD4, then move into the nucleus, where activating of transcription of the hepcidin gene occur (HAMP) (Wang & Cherayil, 2009).

Serum iron levels can also stimulate hepcidin expression independently of BMP6. In this mechanism, hepatocyte transferrin receptors 1 and 2 (TfR1 and TfR2), along with HFE, act as extracellular iron sensors that detect circulating levels of transferrin-bound iron. Because HFE compete with transferrin for binding to TfR1, it associates with TfR1 when serum iron is low (Cappellini et al., 2020).

As serum iron levels rise, transferrin-bound iron ($Tf\text{-}Fe^{2}$) binds to TfR1, displacing HFE. The freed HFE then associates with TfR2. The resulting HFE/TfR2 complex interacts with hemojuvelin (HJV), leading to activate BMP signaling pathway, and stimulate hepcidin production, when there is HFE deficiency, hepcidin decrease, and leads to hereditary hemochromatosis (Ginzburg, 2019), Figure 2.5.

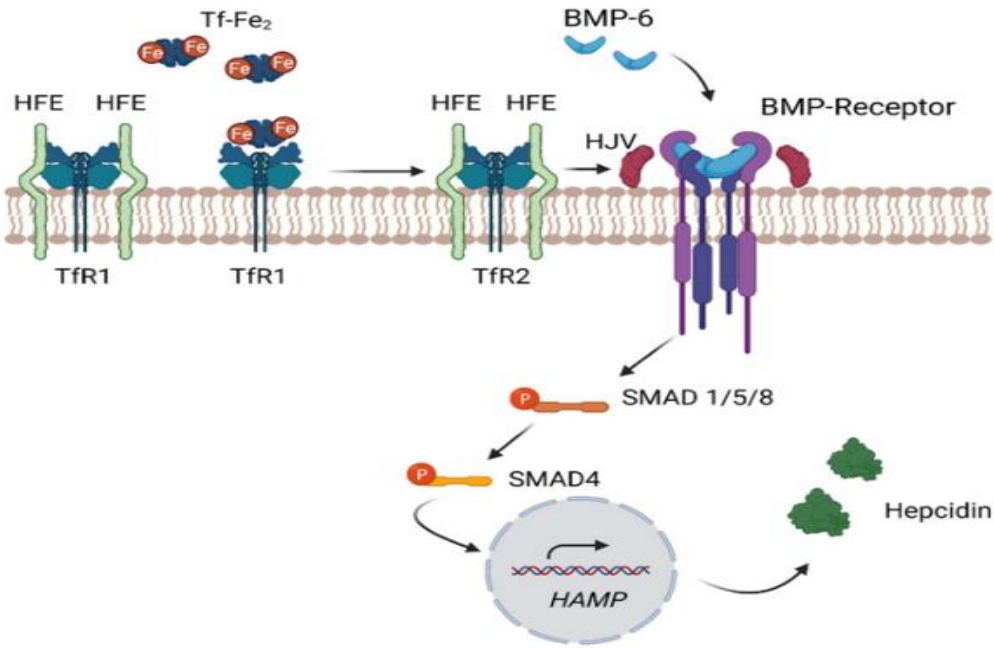

Figure 2.5 Regulation of Hepcidin Expression by BMPs and TfRs (Vogt et al., 2021b).

Conversely, when iron level decrease, HFE binds TFR1, then activates matriptase-2, a serine protease encoded by TMPRSS6 gene (Papanikolaou et al., 2004). Matriptase-2, which mainly expressed in liver, cleaves membrane-bound HJV, leading to release soluble HJV fragments, that antagonize BMP-6 signaling by competing with membrane-HJV for the BMP ligand, and reducing hepcidin transcription so lowering circulating hepcidin levels, to allow greater amount of available iron (Nai et al., 2011), Figure 2.6.

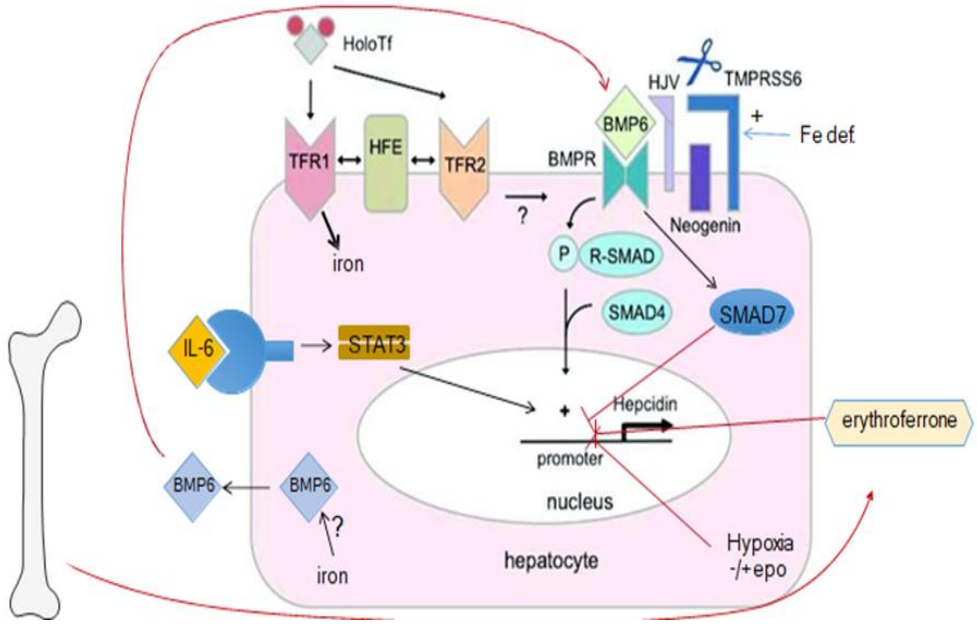

Figure 2.6 Regulation of systemic iron homeostasis (Hoffbrand, n.d.).

Hepcidin Regulation by Inflammation

Inflammatory cytokines, mainly the Interleukin-6 (IL-6), activate hepcidin by STAT3 mediated signaling, this signaling cascade activates hepcidin production, by activating JAK1/2, which in turn phosphorylate STAT3 (Wrighting & Andrews, 2006).

Once phosphorylated, STAT3 moves to the nucleus, and attaches to the hepcidin promoter, upregulate hepcidin expression, leading to inhibition of iron recycling from macrophages, and reduction in iron absorption from intestine, primarily via hepcidin binding to and inactivating ferroportin-1, the only known iron exporter within human cell membrane (Khan et al., 2018) Figure 2.7.

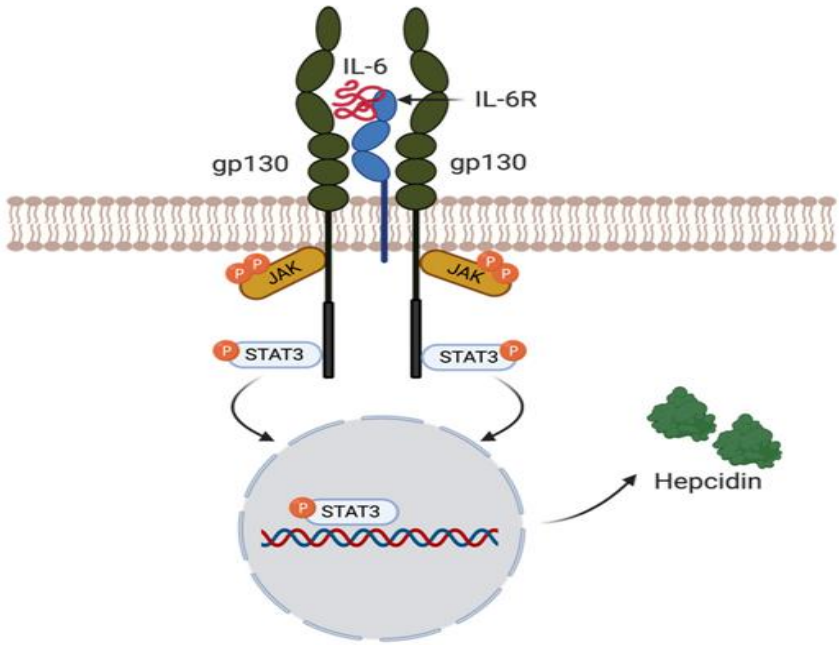

Figure 2.7 Inflammation triggering hepcidin expression (Vogt et al., 2021b).

Stimulatory and inhibitory signals of hepcidin regulation

Hepcidin production is controlled by balance of signals that either stimulate or suppress its expression. In the liver, hepatocytes produce hepcidin, along with another regulatory proteins such as hemojuvelin (HJV), transferrin receptor 2 (TfR2), and HFE. High levels of circulating iron and inflammatory signals both promote hepcidin synthesis, iron activates SMAD signaling, while inflammation activates the STAT3 pathway (Hoffbrand, n.d.).

On the other hand, hepcidin production is reduced when plasma iron levels are low, when erythropoiesis increases, include ineffective erythropoiesis, or during hypoxia. These inhibitory effects are largely mediated by proteins such as matriptase-2 and erythroferrone (ERFE). Once released, hepcidin binds to the iron exporter FPN, triggering its degradation. This reduces dietary iron absorption, limits iron release from macrophages, and restricts iron export from intracellular stores (Hoffbrand, n.d.) Figure 2.8.

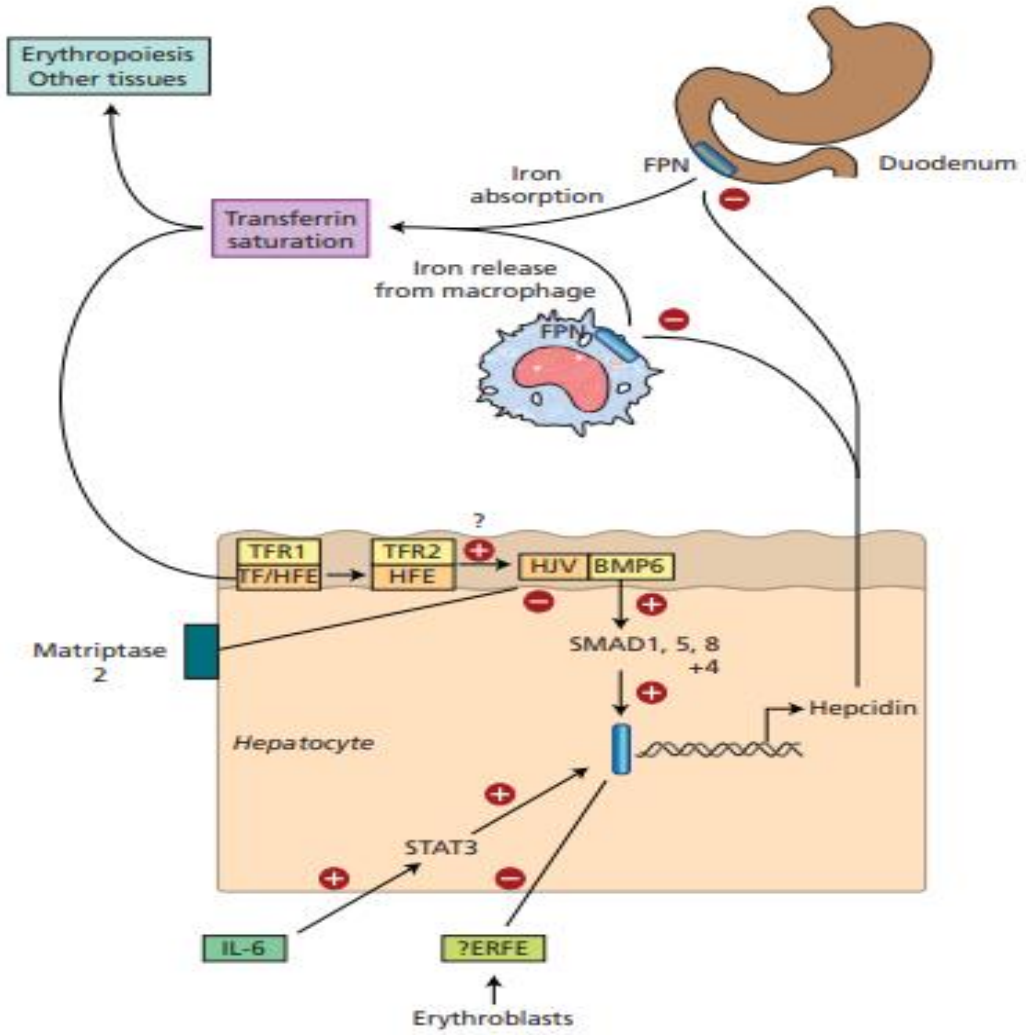

Figure 2.8 Stimulatory and inhibitory signals of hepcidin regulation (Hoffbrand, n.d.)

Matriptase-2 processing

Matriptase-2 is a transmembrane protein, with short cytoplasmic tail. The protein extracellular portion, includes two CUB domains, three repeats of LDLR, prodomain with activation site for the protease, and a C-terminal serine protease domain (Velasco et al., 2002). Homozygous or compound heterozygous mutations in TMPRSS6 in humans, as well as homozygous gene inactivation in mice, lead to a significant increase in hepcidin expression and iron-deficiency anemia (Folgueras et al., 2008).

BMP, HJV, SMAD1, or IL-6 all activate Hepcidin, while it is inhibited in HepG2 cells by matriptase-2, the mutations lead to IRIDA in people, which is defined by elevated hepcidin despite iron deficiency (Finberg, 2009).

Protein function is disrupted by nonsense, frameshift, splice-site, and missense mutations (e.g., G442R, D521N, R774C). Research utilizing mice models in which the catalytic domain removed, and wild-type have variations in transfected cells, they demonstrated suitable cell-surface transport and ER localization, because there was no autocleavage, mice models had higher surface levels of hepcidin (Pagani et al., 2008).

Genetic Modulation of Hepcidin by TMPRSS6

The functional effects of the TMPRSS6 (matriptase-2) rs855791 variant (MT2736A vs. MT2736V) on hepcidin regulation, was examined, in vitro analyses showed that both variants were expressed at similar levels on the cell surface, but MT2736A inhibited hepcidin promoter activity better than MT2736V, particularly in low concentrations, and have slightly higher protease activity. These results indicate that rs855791 is a functional variant, with MT2736V representing some differences associated with more hepcidin production, leading to reduced iron absorption (Nai et al., 2011).

The in vitro findings were validated in a subset of 545 healthy, unrelated individuals from the “Val Borbera” cohort. Serum hepcidin levels, normalized to ferritin and transferrin saturation, were significantly lower in MT2736AA homozygotes compared with MT2736VV homozygotes, while iron and transferrin saturation were higher in AA carriers (Nai et al., 2011).

These results suggest that TMPRSS6 modulates normal hepcidin responses to both circulating and total body iron, counteracting BMP6-dependent and BMP6-independent hepcidin upregulation. Overall, the study demonstrates that rs855791 has a functional role in regulating protease activity and hepcidin expression in vitro and in vivo, potentially influencing iron homeostasis across populations (Nai et al., 2011).

Hemodialysis

Dialysis, come from Greek words *dia* (through) and *lysis* (separation). It is type of renal replacement therapy, that uses artificial equipment to perform the kidney’s filtering function, to remove excess water, solutes, and toxins from the blood. This help in maintaining the internal balance (homeostasis), among patients who suffering from kidney failure,

whether it occurs suddenly such in cases of acute kidney injury, or failure that develops gradually in chronic kidney disease. Dialysis could be as temporary treatment until the kidney function recovers, or a transplant is possible, or as a long-term option, for those patients who cannot undergo transplantation (Pereira, 2000).

The need for renal replacement therapy (RRT), depends on ESRD prognosis, and how early the CKD detected. Identifying patients with worsening kidney function, as (low eGFR), significant proteinuria, or previous acute kidney injury (AKI), help plan RRT in advance, and reduce the number of emergency cases of dialysis. Patients at high risk of ESRD should receive physical and psychological preparation, with good level of education about treatment options, because early planning prevents complications, like catheter malfunction, infection, or bleeding, which can increase mortality (Pereira, 2000).

A study reported that main causes of kidney failure between haemodialysis patients in Somalia were hypertension (33.1%) and diabetes mellitus (27.6%). Other causes included unknown etiology (24.4%), glomerulonephritis (7.1%), obstructive uropathy (3.8%), renovascular hypertension (1.6%), and some few cases of neurogenic bladder, polycystic kidney disease, and congenital or hereditary disorders (0.8%). this highlights the importance of preventing and managing hypertension and diabetes early to reduce the growing burden of kidney failure (Rage et al., 2023).

Anemia among CKD patients

Anemia is serious complication in haemodialysis (HD) patients, it is a strong predictor of mortality within patients (Robinson et al., 2005). Most of patients with CKD require erythropoiesis-stimulating agents (ESAs), to manage the anemia, and it is common among them to be resistance to this therapy (Chung et al., 2023). In HD patients, anaemia is often associated with inflammation and oxidative stress linked to the uraemic syndrome (Guerrero et al., 2025).

Studies reported that CKD patients often require higher ESA dose to maintain recommended hemoglobin target, about 11 g/dL (Koulouridis et al., 2013). In many cases, the decreased responsiveness is related to overt or subclinical inflammation (Y.-Z. Liu et al., 2017a).

In a study about HD patients, that had followed for months, researchers found that patients with hemoglobin levels lower than 11 g/dL, had significantly higher cardiovascular

and mortality risks, and that those patients had higher ESA resistance, also they had markers of inflammation and sometimes malnutrition (Toto, 2006).

Patients with the highest ESA resistance showed lower albumin, higher C-reactive protein (CRP) and interleukin-6 (IL-6) levels. IL-6, in particular, was a strong predictor for ESA resistance. The study concluded that patient's responsiveness to the ESA is important factor for prognosis of morbidity and mortality (Y.-Z. Liu et al., 2017b).

Another related study investigated the effect of the TMPRSS6 rs855791 polymorphism (p.A736V) on the HH clinical expression, which is mostly caused by homozygous HFE p.C282Y mutation. The p.736V allele, which cause higher hepcidin, was underrepresented among HH patients and associated with a protective effect against the disease. Specifically, those with the p.736V/V genotype had a lower risk of HH compared with those carrying the p.736A allele, independently of age, sex, and HFE genotype (Pelusi et al., 2013a).

Further analysis in male C282Y+/+ patients without confounding factors such as chronic hepatitis or alcohol abuse, showed that p.736V allele was negatively associated with cirrhosis and reduced the cumulative incidence of hepatocellular carcinoma. These results suggest that TMPRSS6 rs855791 variant acts as a genetic modifier of HH, and influence both iron overload severity and liver complications (Pelusi et al., 2013a).

Hepcidin levels within CHD

Hepcidin levels are typically elevated among CHD, because hepcidin functions as an acute phase reactant (APR). This elevation is further affected by reduced glomerular filtration and the presence of subclinical inflammation, both common in CHD. This usually make these patients suffering from reduced transferrin saturation, which limits iron availability for erythropoiesis. While ferritin levels, another APR, tend to increase (Y.-Z. Liu et al., 2017b).

ESAs and intravenous iron are typically used to treat anemia in CHD patients. The high levels of circulating hepcidin raise the dosages required for both treatment, which can result in excessive iron storage from longterm supplementation (Y.-Z. Liu et al., 2017b).

The significance of hepcidin regulation in CHD patients has been highlighted by stu dies showing that methods targeted at reducing hepcidin levels can enhance anemia control and raise the likelihood of survival (Valenti et al., 2012).

The p.A736V amino acid substitution caused by polymorphisms in the TMPRSS6 gene, specifically the rs855791 (C>T) variant, has significant effects on iron metabolism. Because TMPRSS6 is less effective in hepcidin transcription inhibiting, this particular polymorphism has linked to less amount of serum iron levels and higher hepcidin concentrations in healthy persons. The A736V variation affects availability of iron, by limiting iron absorption and mobilization, due to insufficient hepcidin suppression (Traglia et al., 2011).

Additionally, prior research has shown that the A736V polymorphism can affect the clinical manifestation of HH, by reducing the degree of iron overload which brought on by HFE gene mutations (Nai et al., 2011).

This modulatory effect makes a balance between TMPRSS6 and HFE in controlling iron stores in the body, indicating that genetic variation at the rs855791 locus may have protective or modifying effects in people who are predisposed to iron-loading disorders in addition to affecting baseline iron parameters in healthy populations (Valenti et al., 2012).

The A736V polymorphism in the TMPRSS6 gene has been linked to altered iron status, and elevated hepcidin levels in numerous prior studies, but, its precise impact in CHD patients, who already show high hepcidin concentrations and disrupted iron metabolism, has not been well investigated.

Most research focused on healthy individuals or patients with HH, leaving a significant gap in our understanding of how A736V polymorphism interacts with anemia management in the CHD population, especially on Palestine.

Investigating whether the A736V variant contributes further to hepcidin elevation in these patients may provide critical insights that could help in optimizing iron supplementation, ESA dosing strategies, improving anemia control and patient outcomes in this vulnerable group.

Chapter Three: Materials and methods

3. Materials and methods:

In this chapter, the research methodology is given with details of the study design, study participants and setting, ethical issues that were considered, inclusion and exclusion criteria, data gathering procedures, blood sample collection, hepcidin measurements, DNA extraction and quantification, genotyping of TMPRSS6 A736V polymorphism using allele specific PCR and statistical analysis.

3.1. Study design:

This study was conducted in the form of a quantitative, case control study at Dr. Khalil Suleiman Hospital in Jenin, Palestine. The case group included adult patients who have Chronic hemodialysis Disease (CHD). The control group comprised of healthy people age and sex strictly matched and carefully screened to eliminate any history of renal disease, anemia, or preexisting iron-related diseases. The particular methodology was chosen so that the direct, comparative analysis of the CHD cohort with the healthy control population could be conducted. This would play a crucial role in assessing the possibility of the variation of iron metabolism and the regulation of hepcidin related to the TMPRSS6 rs855791 (p.A736V) genetic polymorph. The case-control design aligns well in exploring the genetic association and biomarker differences and it provides an effective way to determine the association between the genetic variant and the primary outcome measures, which are changes in the hepcidin and iron parameters.

3.2. Study Participants and setting:

It was a single-center and quantitative, case control study. The patients were informed of the aim and purpose of the study. They ensured that their identity was not known by anyone except the researcher. The information would be safely encrypted, utilized in research, and stored in a password-protected file folder that would not be readily available to any other individual other than the authorized researcher. They also received information that it was entirely voluntary and they could decline. The participants were given liberty to pull out of the research without being subjected to any consequences. The informed consent written

form was given to the consenting patients in an Arabic copy. They were then asked to participate in a face-to-face interview to complete a questionnaire.

The overall population of the study was two different cohorts a case group which consisted of fifty patients on maintenance chronic hemodialysis and were recruited at Dr. Khalil Suleiman Hospital in Jenin Palestine and a control group of fifty healthy people. The case group was carefully matched with the control group in terms of age and sex.

3.3. Ethical Considerations:

This work was accomplished in line with the principles of the Declaration of Helsinki. Approval of the Institutional Review Board (IRB) of Arab American University (AAUP) to undertake the present study was provided on September 10, 2024, and can be located in the archive number R-2024/A/142/N (Appendix 1). Another one, the legal authorization was received in the form of the letter of facilitation of the Palestinian Ministry of Health (MOH) dated September 21, 2024, the aim of which was to perform laboratory work with the basis of providing blood samples and access to patient laboratory data during their registration (Approval Number: 2024/2481/162; Appendix 2). A structured questionnaire in English and Arabic was also utilized in the research tool to gather documentation relative to particular data in the medical history including the primary causes of renal failure, dialysis duration, and smoking status as well as demographic data (Appendices 3,4). The analysis was performed in the Arab American University- Palestine, Faculty of Allied Medical Sciences, 2 nd floor room AMS-B 101 research lab.

3.4. Study Population: Inclusion and Exclusion Criteria:

A total of 50 patients with CHD were enrolled in the study. The control group, 50 subjects, is from apparently healthy people. Inclusion criteria of the case group: patients were under regular maintenance hemodialysis three times a week with a minimum of six months and in a stable condition at the time of sample collection. The exclusion criteria were acute infection, inflammatory diseases, severe iron deficiency (serum ferritin < 30 ng/mL), or severe systemic inflammation (C-reactive protein [CRP] level was more than 1 mg/dL). These strict criteria were applied in order to reduce the confounding effects that were unrelated to the genetic polymorphism being examined.

The control group participants were recruited from the local community or participants who presented for non-renal-related illnesses. Prior to enrollment all potential

control subjects had a thorough medical examination to ensure they did not have chronic renal disease, anemia or any other condition that is known to modify iron metabolism. In order to improve the statistical power and minimize the demographic heterogeneity, each control participant was individually paired with a case patient based on age (within a tolerance of ± 3 years). This strict procedure of selection and matching was planned to provide the comparability of the two groups to ensure that the data may be accurate revealing the relationship among TMPRSS6 A736V polymorphism, hepcidin levels, and iron metabolism.

3.5. Data Collection and Consent:

CHD patients meeting the set inclusion criteria were recruited prospectively in Dr. Khalil Suleiman Hospital, Jenin, Palestine. All subjects signed informed consent (described in Appendix 5) beforehand. Eligibility was ensured by retrieving pertinent medical history and records of patient's files. Data was collected from clinical records, laboratory assays, and genetic analysis to provide whole view of hematological indices, iron status, and inflammatory markers, strengthening the ability to identify potential correlations and control related factors such as erythropoietin therapy or intravenous iron supplementation. The standardized questionnaire was used to gather demographic and clinical information that contained age, sex, dialysis duration, hypertension and diabetes history, smoking habits, C-reactive protein (CRP) levels, and main cause leading to dialysis.

3.6. Blood Sample Collection:

After every participant filled out the questionnaire, venous blood samples were collected. Two vacutainer tubes were used to collect the blood: About 3 milliliters of venous blood were collected in tube that contained Ethylene Diamine Tetra Acetic Acid (EDTA) and 3 milliliters in a plain tube. Prior to testing, serum and EDTA whole blood samples were stored at -80 °C and 4 °C, respectively.

The blood sample was taken, prior to any invasive procedure, including insertion of intravenous lines, or fibrinolytic, antithrombotic or antiplatelet medicines. The level of hepcidin in the serum sample was determined by the use of ELISA kit. The anticoagulated EDTA tube was used in isolating DNA to assess the TMPRSS6 rs855791 (p.A736V) polymorphism.

Venous blood samples were taken from all study participants, including patients with Chronic Hemodialysis (CHD) and a healthy control group. To minimize pre-analytical variation in the measured parameters, samples of the CHD group were collected in the morning, right before the hemodialysis session. Strict washout was adhered to, so that there could be no variation of iron status and hepcidin: the samples were taken at least three days post-erythropoietin (EPO) injection and a week post-intravenous (IV) iron injection where possible. For CHD patients, samples were collected from the established dialysis lines as soon as the patient was connected to the dialysis machine. This approach reduced patient discomfort from not having to have another venipuncture.

Routine hospital laboratory records were retrospectively accessed to obtain clinical and biochemical parameters and create a comprehensive baseline profile of each of the participants. These data included hematological indices, iron status markers and renal function tests.

The Hematological indices were acquired as the Complete Blood Count (CBC) results, which had hemoglobin (Hb), mean corpuscular volume (MCV), hematocrit (Hct), red blood cell count (RBCs), and red cell distribution width (RDW). Markers of iron status used were ferritin, transferrin saturation (TS), serum iron and total iron-binding capacity (TIBC). The markers of renal functioning included blood urea nitrogen (BUN) and serum creatinine.

3.7. Hepcidin Measurement:

The Hepcidin levels were measured in serum with the use of commercially available Human Hepcidin ELISA kit (Invitrogen, Thermo Fisher Scientific, Catalog #EEL073, Austria), according to manufacturer instructions using the ELISA analyzer (HumanReader Single Microtiter Strip, Human, Germany). The assay is intended to be used in quantitative determination of hepcidin in different biological fluids such as serum and plasma.

Assay employs a solid-phase sandwich Enzyme-Linked Immunosorbent Assay. The wells of the provided microplate are pre-treated with target-specific capture antibody. After addition of samples, the hepcidin immunoprecipitated with this capture antibody. This is then followed by the conjugation of a second conjugated detection antibody that, in turn, binds to another epitope on the target and forms a fraction of the sandwich complex. After a step of washing to get rid of unbound material, there is a step of adding a substrate solution. The interaction between the substrate and the enzyme-antibody complex produces a signal that

can be measured whose intensity is directly proportional to the concentration of hepcidin in the original sample. The kit can only be used in research (RUO) but not in a diagnostic procedure.

The seven calibrators, i.e., 62.5, 125, 250, 500, 1000, 2000 and 4000 ng/mL, were used to create a standard curve (where the zero well was taken as a blank (0 ng/mL). All incubation procedures were performed at 37 ℃.

The technical manual of the kit was followed precisely, and the procedure in detail started with the antigen binding phase. The samples and the prepared standards were transferred to the pre-coated microplate wells and allowed to incubate after 90 minutes and allow the hepcidin analyte to bind with the immobilized capture antibody. After this initial incubation, a biotinylated detection antibody was then added and left to incubate at 60 minutes. Three wash cycles were then carried out to remove unbound reagents.

A Horseradish Peroxidase (HRP) conjugate was then added and incubated after 30 minutes after which a thorough five-wash cycle was taken to remove all the non-specifically bound conjugate. The substrate solution was then added to initiate the colorimetric reaction and left to react over 15 minutes. The stop solution was added to the enzymatic reaction and the color changed to yellow. The degree of the final yellow color is in direct proportion to the concentration of hepcidin in each of the wells.

Lastly, optical density (OD) of the wells was determined at 450 nm with the aid of microplate ELISA reader. Interpretation of the standard curve generated was the determination of the hepcidin concentrations in the unknown samples. This method assures precise standard concentrations, and colorimetric evaluation, it is also ensured accurate and reproducible quantification of serum hepcidin levels for all participants, with clear differentiation of sample concentrations based on the observed color intensity in each well.

3.8. DNA Extraction and Quantification:

Genomic DNA was isolated from the buffy coat fraction using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), strictly following the manufacturer's protocol. The extraction commenced by combining µL of Proteinase K with $200 200µL of blood. Subsequently, 200µL of Buffer AL (lysis buffer) was added, and the mixture was incubated at 56℃ for 15 minutes. Lysis was completed by vortexing the mixture after the addition of

250µL of absolute ethanol. The lysate was then incubated at room temperature (15-25℃) for 5 minutes.

The resulting lysate was transferred to a QIAamp MinElute column and centrifuged at 8000 rpm for 1 minute. The column was then washed sequentially with Washing Buffer AW1 and Washing Buffer AW2, each performed twice, to remove protein contaminants, cell debris, and residual lysis buffer as well as to help maintaining DNA binding to the silica column while removing impurities. For the final wash, 500 mL of 96-100% ethanol was added to the column and centrifuged at 8000 rpm for 1 minute. Finally, the purified DNA was eluted by adding 70µL of the kit's AVE buffer, (which composed of Tris to stabilize the DNA, and EDTA to protect DNA by chelating divalent ions (Mg^{2+}), which inhibits DNases), to the column and collecting the flow-through into a sterile microcentrifuge tube. The isolated DNA was stored at -20 ℃ until further use.

The concentration and purity of the extracted genomic DNA were assessed using a NanoDrop spectrophotometer (IMPLEN, Germany). Specifically, a 1µL aliquot of the extracted DNA sample was measured following a blank reading using 1µL of AVE elution buffer. DNA purity was estimated by calculating the OD260/OD280 absorbance ratio. A ratio within the acceptable range of 1.8-2.2 was considered indicative of low protein contamination and suitability for downstream molecular analysis.

3.9. Genotyping:

The TMPRSS6 rs855791 (p.A736V) polymorphism was genotyped using allele-specific PCR (AS-PCR) with primers specific for the C and T alleles (hylabs, Ltd., Israel). The primer sequences are shown in table 3.1: common reverse primer 5’-GATGTGAGCAAAGGGCCAGAC-3’, allele C-specific forward primer 5’-CACAGGACCTGTGCAGCGAGGC-3’, and allele T-specific forward primer 5’-CACAGGACCTGTGCAGCAAGGT-3’, as previously described (Valenti et al., 2012). The specificity of primer sequences was examined using UCSC In-Silico PCR (http://www.genome.ucsc.edu) and NCBI Primer-BLAST.

Table 3.1. Primer sequences.

Primer	Sequence
common reverse primer	5’-GATGTGAGCAAAGGGCCAGAC-3’
C-specific forward primer	5’-CACAGGACCTGTGCAGCGAGGC-3’
T-specific forward primer	5’-CACAGGACCTGTGCAGCAAGGT-3’

To guarantee that the PCR product was free of contamination, a PCR mixture devoid of a DNA sample was utilized as a negative control. The PCR was performed in a total volume of 25µl using 100 ng of DNA, 1x reaction buffer, 1.5 µM MgCl2, 0.5 µM dNTPs and 0.5 µM Taq polymerase (Promega, Madison, USA). DNA was amplified using a Biometra Tadvanced Thermal Cycler (analytikjena, Fullerton, USA). The respective reaction mixtures were made up with 12.5 µL of a ready-to-use master mix, 3 µL of genomic DNA template, and 7.5 µL of nuclease-free distilled water. The primer composition varied by allele: The C-specific reaction had 1 µL of the C-specific forward primer and 1 µL of a universal reverse primer and the T-specific reaction used the T-specific forward-specific primer in place of the C-specific forward-specific primer as shown in Table 3.2.

Table 3.2. Amount of each used component in PCR mixture.

Component	Amount
Ready-to-use master mix	12.5 µL
Genomic DNA template	3 µL
Nuclease-free distilled water	7.5 µL
C- or T- specific forward reaction primer	1 µL
Universal reverse primer	1 µL

Thermal cycling protocol was performed in the following manner: a denaturation of 95 o C of 5 minutes, then 30 rounds of denaturation at 95 o C of 45 seconds, annealing at 63 o C of 45 seconds, and extension at 72 o C of 45 seconds. Another extension was carried out

at 72 o C and 5 minutes. The anticipated product of this amplification strategy is a PCR product of 249 base pairs (Valenti et al., 2010).

To establish appropriate amplification and visualization of the allele-specific products, the resulting PCR amplicons were performed using 2% agarose gel with Ethidium bromide in 1x TBA buffer (Promega, Madison, USA) and electrophoresed using ultraviolet transilluminator documentation system (Uvitec, Cambridge, UK). The electrophoresis was performed with 40 minutes at 94 volts. The amplification of the target allele was verified by the fact that the 249 bp band was present as expected.

3.10. Statistical Analysis:

Data was analyzed using SPSS software, details will be shown on the results chapter. Briefly, normality of continuous variables was first assessed using both the Kolmogorov-Smirnov and Shapiro-Wilk tests. Based on these results, variables that met the assumption of normal distribution were analyzed using parametric tests, whereas non-normally distributed variables were examined using non-parametric alternatives. Descriptive statistics were presented as mean ± standard deviation for normally distributed data and as median with interquartile range for non-normal data. Graphical presentation included histograms and Box plots for normality assessment, as well as boxplots to illustrate differences between genotype groups and between patients and controls, Results were considered significant when p was lower than 0.05 (two-tailed).

Chapter Four: Results

4.1. Descriptive Statistics:

The baseline characteristics of all the participants were initially analyzed to give a clear understanding of the study population and to assure the relevant comparability between groups. This part includes a summary of the demographic and clinical characteristics of both Chronic Hemodialysis (CHD) patients and healthy controls, such as the age distribution, sex ratio, smoking habits, as well as comorbidities. These descriptive statistics are necessary in the interpretation of further analyses since variations in these baseline variables can affect iron metabolism, hepcidin expression, or genetic variation.

Table 4.1 presents the baseline characteristics of the study participants. The sample size was 50 CHD patients and 50 healthy controls. The Shapiro-Wilk test was used to test the normality of continuous variables. Variables normally distributed (age, BMI, creatinine, hemoglobin, RBC count, hematocrit, TIBC) are provided as mean ± standard deviation (SD). Continuous variables (skewed: MCV, RDW, serum iron, transferrin, ferritin, hepcidin, and dialysis session duration) are expressed as median and range (minimum-maximum). The categorical variables (gender and smoking status) are expressed in absolute numbers.

Table 4.1: Baseline characteristics and Biomarkers of the study participants.

Variable	**CHD Patients (n = 50)**	**Controls (n = 50)**	**p-value**
Gender (Female)	16 (out of 50)	24 (out of 50)	0.10
Age (years)	52.7 ± 13.5	51.5 ± 14.0	0.66
BMI (kg/m²)	25.26 ± 5.73	24.5 ± 5.5	0.911
Active smoking	20 smokers 30 non-smokers	16 smokers 34 non-smokers	0.41
Creatinine (mg/dl)	8.9 ± 2.77	0.95 ± 0.15	0.001
Hb (g/dl)	9.66 ± 1.06	13.5 ± 1.5	0.001
RBC (M/µl)	3.46 ± 0.39	4.7 ± 0.5	0.001
HCT (%)	28.82 ± 3.16	42.0 ± 4.0	0.001

Variable	CHD Patients (n = 50)	Controls (n = 50)	p-value
MCV (fL)	88.85 (63.2-101)	91.8 (85-102)	0.001
RDW (%)	15.05 (13-19.8)	13.0 (11.2-15.2)	0.001
Serum iron (µg/dl)	47 (14-244)	90.0 (35-150)	0.001
Transferrin (mg/dl)	141.5 (55-214)	280.0 (190-390)	0.001
Ferritin (ng/ml)	478 (54–1840)	80.0 (20–150)	0.001
TIBC (µg/dl)	185.3 ± 35.8	350.0 ± 80.0	0.001
Hepcidin (ng/ml)	525 (150-1240)	78.5 (26–120)	0.001
Hemodialysis session duration (hours)	3.0 (2–3.5)	—	—

4.2. Comparison of Serum Hepcidin Levels Between CHD Patients and Controls:

Mann-Whitney U test was used to compare serum hepcidin levels in CHD patients and healthy controls because the data were not normally distributed. Figure 4.1 indicates that the difference in the median levels of hepcidin between CHD patients and controls was significant (525 [150 - 1240] ng/ml vs. 78.5 [26 - 120] ng/ml, respectively, Mann-Whitney U test, $p < 0.001$).

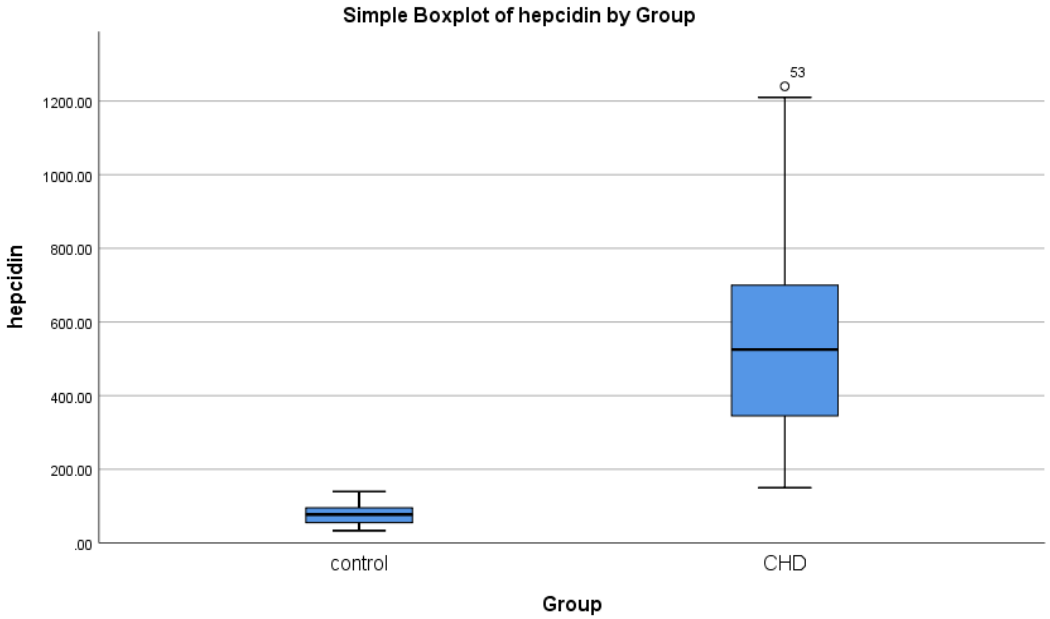

Figure 4.1. Boxplot Mann–Whitney U test, to compare serum hepcidin levels between CHD patients and controls.

4.3. Frequency distribution TMPRSS6 variants in patients and controls:

The TMPRSS6 A736V genotypes (CC, CT, TT) were determined and evaluated between hemodialysis (CHD) and healthy controls. The most common genotype overall, as indicated in Figure 4.2, was CT, with a prevalence of 66 % of participants (44 controls and 22 CHD patients). The CC genotype was found in 23% of the participants with 5 controls and 18 CHD patients, whereas the TT genotype was least with only 11% of the participants (1 control and 10 CHD patients).

The control group was in significant disequilibrium with Hardy Weinberg genotype of TMPRSS6 A736V variant (χ 2 = 29.75, $p < 0.001$). This high divergence indicates a potential issue with the control group, including selection bias, non-random mating or a genotyping error, and should be interpreted with caution the genetic data of the control group. Moreover, the genotype frequencies were significantly different in CHD patients and controls (Pearson $\chi^2 = 22.045$, df = 2, $p < 0.001$).

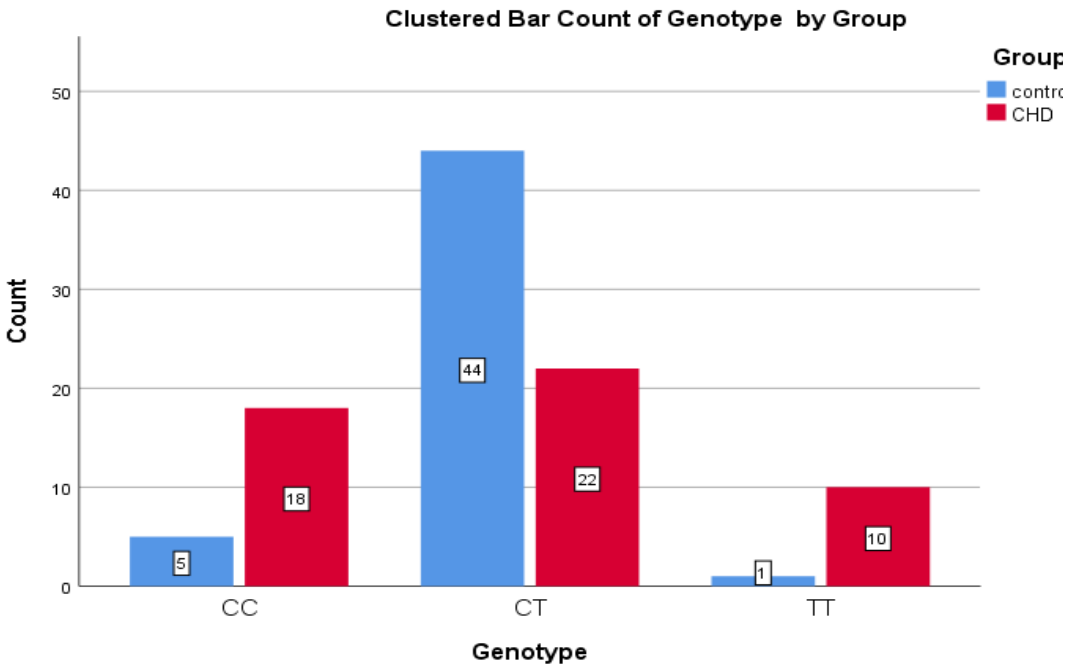

Figure 4.2. Clustered bar chart showing the frequency distribution of TMPRSS6 (A736V) genotypes (CC, CT, and TT) across the two groups (patients and controls).

Table 4.2 shows the allele frequencies of the TMPRSS6 A736 V polymorphism in the overall study population. The C allele was prevalent as compared to the T allele (56 to 44 %).

Table 4.2. TMPRSS6 A736V polymorphism C and T allele frequencies.

Allele	Count	Frequency
C	112	56%
T	88	44%

Figure 4.3 and Figure 4.4, related to the agarose gel electrophoresis results, provide a brief overview of the frequency of each genotype among control and CHD patients, respectively, each revealed a clear single DNA band at approximately 250 bp, as estimated using a 50 bp DNA ladder.

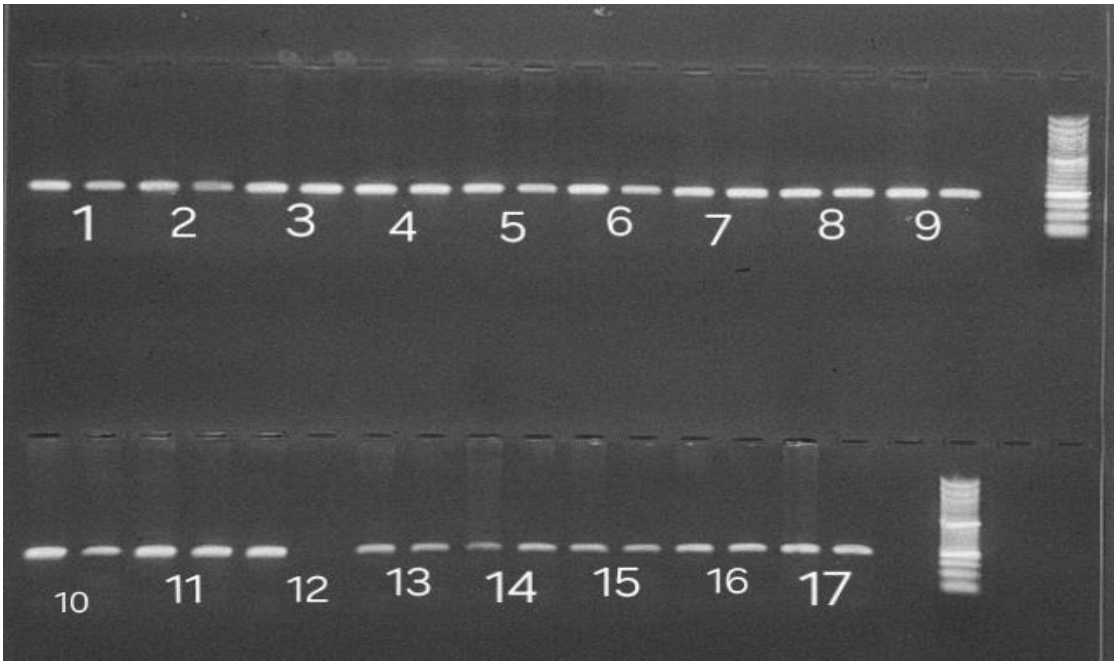

Figure 4.3. Agarose gel electrophoresis of PCR products for the TMPRSS6 (A736V) polymorphism in the control group. The majority of samples exhibited both C- and T-specific bands, indicating a heterozygous CT genotype, while sample 12 showed only the C-specific band (CC homozygous genotype).

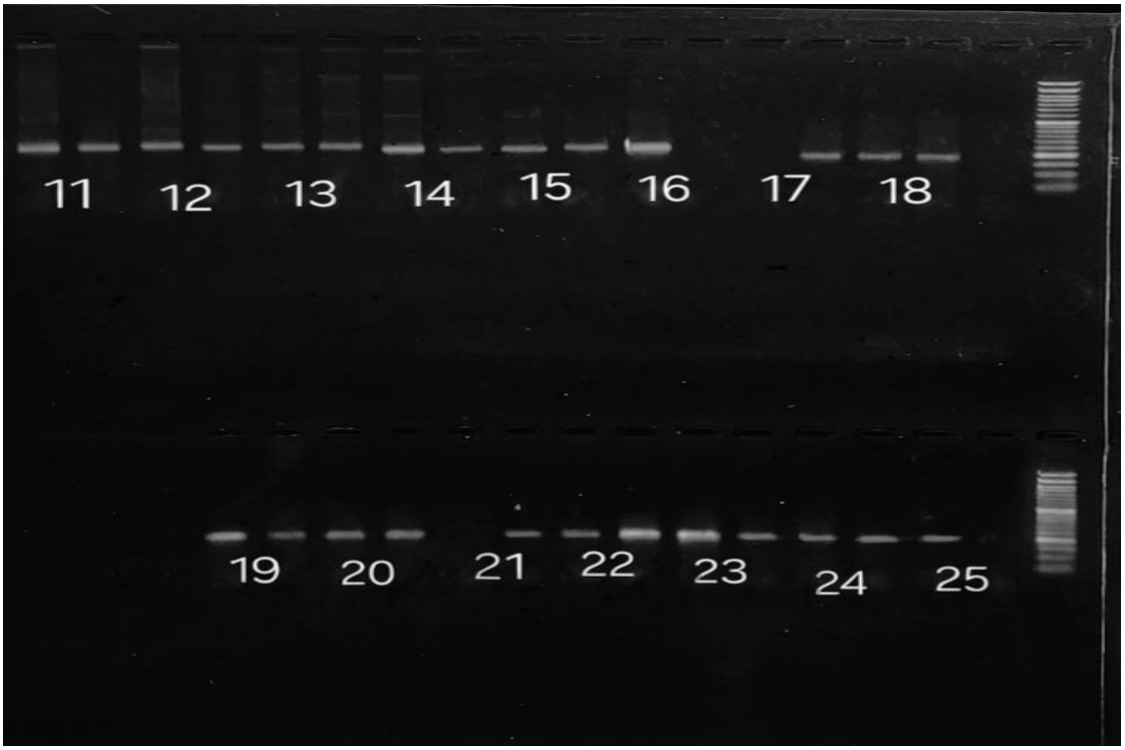

Figure 4.4. Agarose gel electrophoresis of PCR products for the TMPRSS6 (A736V) polymorphism in chronic hemodialysis (CHD) patients. Most samples showed a heterozygous CT genotype, while sample 16 was homozygous CC and samples 17 and 21 were homozygous TT.

4.4. Evaluation of Hepcidin Levels Across TMPRSS6 Genotypes in the Total Cohort:

To determine whether the serum levels of hepcidin varied across the three TMPRSS6 A736V genotypes (CC, CT, and TT), non-parametric Kruskal-Wallis test was used since the values of hepcidin do not follow a normal distribution. It was found that there was a statistically significant difference in the levels of hepcidin in the genotypic groups ($H = 20.626$, $p < 0.001$). The null hypothesis, which postulates that the level of hepcidin is equally distributed across all the genotypes, was therefore rejected, which implied that at least one genotype group does not match with the other genotype groups. Thus, the specific genotype pairs that contribute to the observed

overall difference were identified by post-hoc analyses with the help of proper multiple comparison tests.

The logarithmic transformation of serum hepcidin levels was followed by different distributions of hepcidin in TMPRSS6 genotypes across the controls and CHD group as shown in Figure 4.5. Overall, CHD patients in the CC genotype showed greater median log-hepcidin levels than in the controls, although there were outliers. The CT genotype also exhibited high median in CHD as compared to controls. The TT genotype was monozygotic with only CHD patients exhibiting fairly high levels of log-hepcidin. In general, CHD patients displayed high levels of log-hepcidin within all genotypes as opposed to healthy controls.

The log transformation played a significant role in equalizing the skewed statistics of the serum hepcidin levels, and in stabilizing the variance based on genotype. This change minimized the effect of extreme outliers and made a more precise comparison between CHD patients and healthy controls possible. Using log scale, the slight variations in hepcidin levels by each genotype were more visible, which improved the visibility of the patterns and enabled the use of reliable statistical tests.

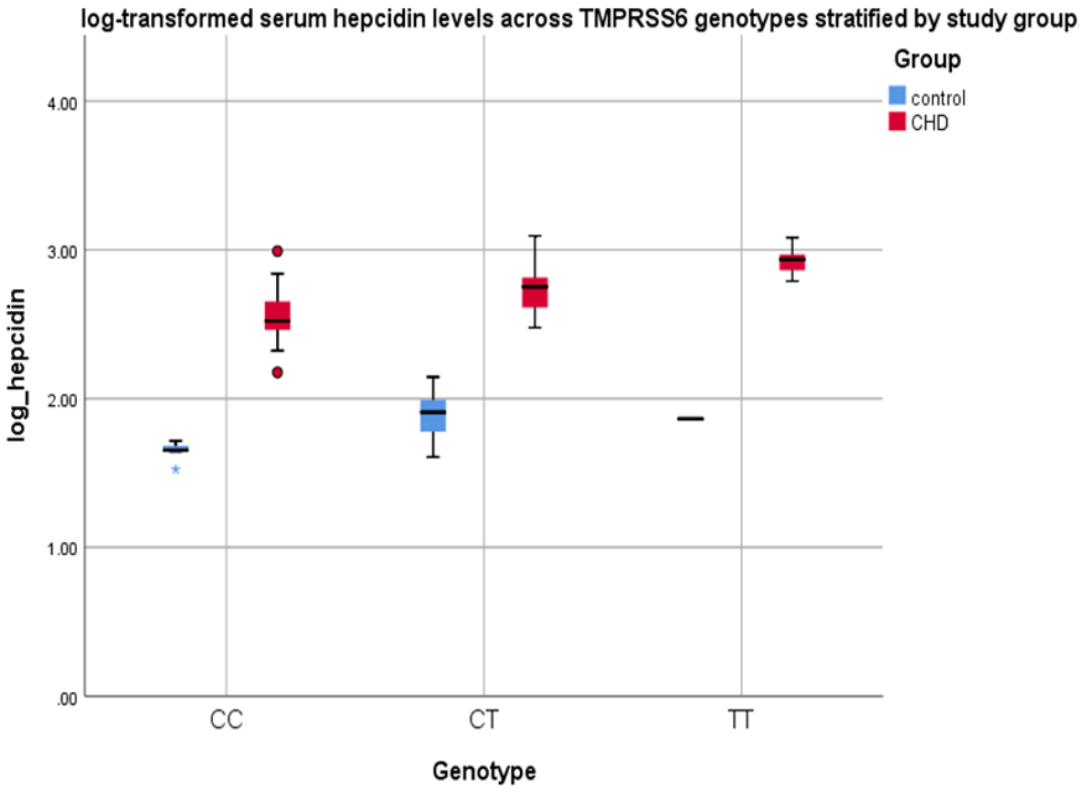

Figure 4.5. Log-transformed serum hepcidin levels across TMPRSS6 genotypes stratified by study group.

4.5. Post-hoc Analysis

4.5.1. Comparison of Serum Hepcidin Levels Across TMPRSS6 Genotypes in in the Total Cohort

Differences in serum hepcidin were evaluated by post-hoc pair-wise comparisons with the Bonferroni correction applied to test differences between three TMPRSS6 A736 V genotypes (CC, CT, TT). The results were significant on the effect sizes between CC and TT (mean difference = -470.07, p = 0.001) and CT and TT (mean difference = -544.25, p = 0.001). These findings imply that hepcidin was elevated significantly in TT carriers as opposed to both CC and CT carriers. No notable distinction was established between CC and CT (mean difference = 74.18, p = 0.673) (Table 4.3).

Table 4.3. Bonferroni test results for hepcidin levels among TMPRSS6 genotypes in the total cohort.

(I) Genotype	(J) Genotype	Mean Difference (I-J)	Std. Error	Sig.	95% CI	
CC	CT	74.18202	60.64191	.673	-73.5542-	221.9182
	TT	-470.06798-*	91.81063	.000	-693.7376-	-246.3983-
CT	CC	-74.18202-	60.64191	.673	-221.9182-	73.5542
	TT	-544.25000-*	81.56256	.000	-742.9532-	-345.5468-
TT	CC	470.06798*	91.81063	.000	246.3983	693.7376
	CT	544.25000*	81.56256	.000	345.5468	742.9532

CI, Confidence Interval

4.5.2. Comparison of Serum Hepcidin Levels Across TMPRSS6 Genotypes in Controls

Post-hoc pair-wise comparisons were used to assess the difference between the serum hepcidin levels of the three TMPRSS6 A736V genotypes in the control group. The difference between CC and CT (mean difference = -34.22, p = 0.011) and CC and TT carriers (mean difference = -61.87, p = 0.009) were significant, which indicates that the levels of hepcidin were lower in CC carriers than in CT and TT carriers. There was no significant difference between CT and TT (Table 4.4).

Table 4.4. Bonferroni test results for hepcidin levels among TMPRSS6 genotypes in the control group.

(I) geno_control	(J) geno_control	Mean Difference (I-J)	Std. Error	Sig.	95% CI	
CC	CT	-34.22233-*	11.13848	.011	-61.8758-	-6.5689-
	TT	-61.87000-*	19.72303	.009	-110.8363-	-12.9037-
CT	CC	34.22233*	11.13848	.011	6.5689	61.8758
	TT	-27.64767-	17.05225	.335	-69.9832-	14.6878
TT	CC	61.87000*	19.72303	.009	12.9037	110.8363
	CT	27.64767	17.05225	.335	-14.6878-	69.9832

CI, Confidence Interval

4.5.3. Comparison of Serum Hepcidin Levels Across TMPRSS6 Genotypes in CHD Patients:

The post-hoc pairwise comparisons indicated that there were significant differences in serum hepcidin levels across all TMPRSS6 A736V genotypes in CHD patients. The hepcidin concentration was found to be lower in CC carriers than in CT (mean difference = -167.83, p=0.026) and TT carriers (mean difference = -466.56, p=0.001). Moreover, hepcidin levels were found to be much lower in CT carriers, in comparison to TT carriers (mean difference = -298.73, p = 0.001), and there was a step-wise growth in hepcidin levels in carriers of the CC, CT, and TT genotypes (Table 4.5).

Table 4.5. Bonferroni test results for hepcidin levels among TMPRSS6 genotypes in the CHD group.

(I) geno_CHD	(J) geno_CHD	Mean Difference (I-J)	Std. Error	Sig.	95% CI	
CC	CT	-167.82828-*	61.13992	.026	-319.6200-	-16.0365-
	TT	-466.55556-*	75.87265	.000	-654.9242-	-278.1870-
CT	CC	167.82828*	61.13992	.026	16.0365	319.6200
	TT	-298.72727-*	73.36791	.001	-480.8774-	-116.5772-
TT	CC	466.55556*	75.87265	.000	278.1870	654.9242
	CT	298.72727*	73.36791	.001	116.5772	480.8774

CI, Confidence Interval

4.6. Correlations of Serum Hepcidin with Clinical Variables in CHD:

A Spearman correlation test was done to check the relationship between serum hepcidin levels and clinical/biochemical variables in CHD patients (n=50). Ferritin (rho = 0.302, p = 0.033) and creatinine (rho = +0.566, p = 0.000) had a positive correlation with hepcidin. Hepcidin did not have a significant correlation with any other variables such as serum iron, transferrin, TIBC, hemoglobin, MCV, RDW, Hct, and RBC (Table 4.6).

Table 4.6. Clinical variables associated with hepcidin levels (Spearman's rho test) in CHD patients.

Variable	R (Spearman's rho)	p-value (2-tailed)	Trend	Significance
Ferritin (ng/ml)	+0.302	0.033	Positive	Significant
Creatinine (mg/dl)	+0.566	0.000	Positive	Significant
Serum iron (µg/dl)	-0.023	0.874	Negative	Ns
TIBC (µg/dl)	-0.014	0.921	Negative	Ns
Transferrin (mg/dl)	-0.077	0.597	Negative	Ns
Hemoglobin (g/dl)	-0.180	0.212	Negative	Ns
MCV (fl)	-0.190	0.187	Negative	Ns
RDW (%)	+0.137	0.344	Positive	Ns
Hct (%)	-0.202	0.159	Negative	Ns
RBC (M/µl)	-0.250	0.080	Negative	Ns
BUN (mg/dl)	-0.050	0.731	Negative	Ns

Subsequently, a Mann Whitney U test was conducted to show a comparison of the hepcidin levels in blood serum of males and female patients with CHD. Gender did not show any significant difference in hepcidin distribution ($p = 0.811$), which demonstrates that there was no difference between males and females in the level of hepcidin.

Finally, Mann-Whitney U test was used to determine whether the level of hepcidin varied between categories of smoking status among CHD patients. The findings suggested that there was no statistically significant differentiation in the distribution of hepcidin in the groups ($p = 0.656$). The p-value was greater than the significance level of 0.05, which means that the null hypothesis was not rejected, indicating that smoking status does not significantly influence hepcidin levels in the current sample of CHD patients.

4.7. Underlying Causes of End-Stage Renal Disease:

Figure 4.6 shows the prevalence of underlying causes of end-stage renal disease in hemodialysis patients. The most common cause was diabetes mellitus which represented a significant percentage in cases either on its own or co-morbidly with hypertension. Causes relating to hypertension also played a significant percentage among the study population. Surgical and post-surgical causes, such as drug toxicity,

and complications of post-cardiac surgery were a significant percentage of the cases. The less common causes included hereditary and metabolic ones, including primary oxalosis, and a minority number of patients had an unknown etiology.

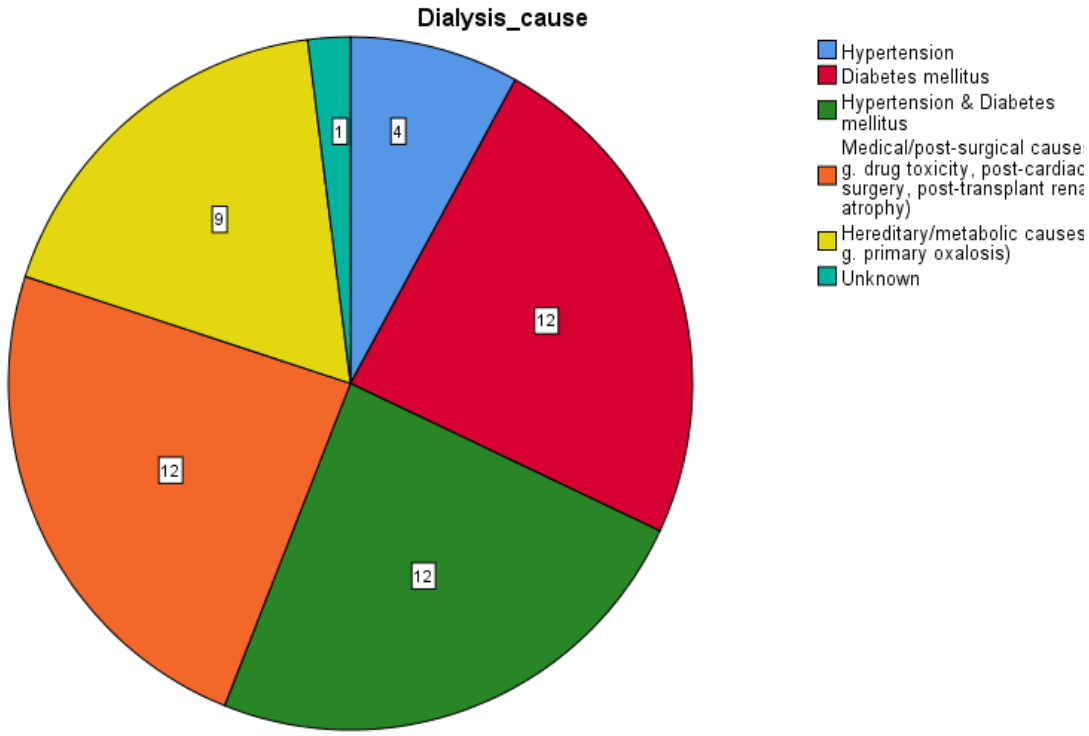

Figure 4.6. Underlying Causes of End-Stage Renal Disease in the CHD patient cohort.

Chapter Five: Discussion

5.1. Discussion:

Management of anemia among patients on chronic hemodialysis is a clinical issue that continues to challenge clinicians owing to the severe alterations in iron homeostasis. These disproportions are facilitated by persistent inflammation, continued blood loss, and erythropoietin therapy (Canavesi & Valenti, 2011). The key pathophysiology is the increase of serum hepcidin that interferes with iron use and induces functional iron deficiency, thus, impairs the erythropoietic efficiency of CHD patients (Babitt & Lin, 2010).

The hemodialysis patients and control group were comparable in terms of age, sex distribution, and body mass index. This similarity reduces the likelihood that differences in hepcidin levels and iron-related biomarkers are confounded by demographic factors, allowing a more reliable interpretation of disease- and genotype-related effects.

According Table 4.1 patients with chronic hemodialysis exhibited lower hemoglobin, red blood cell count, and hematocrit compared to healthy controls as expected, reflecting the well-established anemia of chronic kidney disease. This finding supports the clinical validity of the patient cohort included in the study. Also, the iron profile of CHD patients was characterized by lower serum iron and transferrin levels, accompanied by elevated ferritin concentrations. This pattern is consistent with functional iron deficiency and iron sequestration, which are commonly observed in chronic kidney disease due to inflammation and impaired iron mobilization (Valenti et al., 2012).

Elevated hepcidin levels in hemodialysis patients reflect impaired renal clearance and inflammation-driven upregulation. Increased hepcidin contributes to reduced intestinal iron absorption and iron retention within macrophages, thereby

exacerbating anemia despite adequate or elevated ferritin levels. Relatively uniform duration of hemodialysis sessions among patients minimizes treatment related variability, suggesting that observed biochemical differences are less likely to be driven by dialysis duration. Taken together, the descriptive characteristics of the study population support the internal validity of the analysis and provide solid basis for interpreting the observed biochemical and genetic associations.

The statistically significant difference in serum hepcidin levels between chronic hemodialysis patients and the control group in this study, is consistent with previously published studies reporting significant alterations in hepcidin regulation among hemodialysis patients compared with healthy individuals (Valenti et al., 2012). These studies highlight the impact of disrupted iron metabolism, chronic inflammation, and erythropoietin therapy on hepcidin expression in this population. In contrast, (Pelusi et al., 2013a) reported no significant difference in hepcidin levels between hemodialysis patients and controls, a discrepancy that may be attributed to differences in study design, sample size, patient characteristics, and iron or inflammatory status. Overall, our findings are similar to existing evidence supporting a significant difference in hepcidin levels in patients undergoing chronic hemodialysis.

Correlation analysis in the present study according to Table 4.6 demonstrated a significant positive association between serum hepcidin and ferritin levels, supporting the established role of hepcidin as a key regulator of iron storage. Ferritin reflects body iron reserves and is also an acute-phase reactant, therefore, its positive correlation with hepcidin is consistent with previous findings linking increased iron stores and inflammatory signaling to hepcidin upregulation (Pelusi et al., 2013a)

In addition, Hepcidin showed a significant positive correlation with creatinine levels (Spearman's rho = 0.566, $p < 0.001$), indicating that higher hepcidin concentrations were associated with greater renal impairment. This

finding is consistent with the role of the kidneys in hepcidin clearance, whereby reduced renal function may lead to decreased hepcidin elimination and subsequent accumulation in circulation. These results are similar to what was published in another study which found that only IL-6, ferritin and serum creatinine significantly correlate with serum hepcidin from seven tested variables, revealed that they can be used to predict the hepcidin level in patients with anemia of chronic disease (Suega & Widiana, 2019). As well as similar to (Troutt et al., 2013) that concluded the direct correlation of hepcidin with creatinine and its inverse correlation with eGFR suggest that hepcidin levels increase as renal function deteriorates, possibly because of decreased hepcidin renal clearance.

Despite the lack of statistically significant correlations between hepcidin and other iron indices or hematological parameters, an overall inverse pattern was observed, suggesting that higher hepcidin levels tend to coincide with reduced iron availability and erythropoietic indices. The absence of significance may be attributed to the complex interplay of inflammation, iron metabolism, and comorbid conditions in CHD patients, As well as the limited sample size. These findings emphasize that hepcidin regulation in CHD is influenced by multiple physiological pathways rather than a single biochemical marker (Babitt & Lin, 2010).

In the present study, serum hepcidin levels did not differ significantly between male and female CHD patients, suggesting that gender does not independently influence hepcidin expression in this cohort. Also smoking status was not significantly associated with serum hepcidin levels in CHD patients. This finding suggests that, within this cohort, smoking may not independently influence hepcidin regulation. The lack of a significant association may be explained by the dominant effect of chronic inflammation and underlying disease processes in CHD, which could overshadow potential modulatory effects of smoking on iron metabolism.

The main hypothesis of the current study was to establish the extent to which the A736V TMPRSS6 polymorphism, an established regulator of hepcidin expression and determinant of iron-restrained erythropoiesis in the normal population (Nai et al., 2011), had a similar effect on hepcidin and erythropoiesis in patients with Chronic Hemodialysis (CHD). The preliminary analysis was carried out on the frequency distribution of TMPRSS6 genotypes and alleles in a patient and control group, which are presented in Figure 4.2 and Table 4.2. The described genotype distribution, including the heterozygous CT genotype as the most common, followed by CC and then TT, is consistent with most of the published studies (Pelusi et al., 2013a).

On the other hand, for a Sudanese cohort, they have a diverging distribution pattern (CC > CT > TT) (Ahmed et al., 2023). indicating the diversity of the genotype frequency across different groups. These variations can probably be explained by the ethnic and genetic heterogeneity of humans due to the recognized linear variation in the allele frequencies of the TMPRSS6 polymorphisms depending on the ancestral background and the population structure.

A higher frequency of the heterozygous CT genotype was also reported in previous Palestinian cohorts, as well as the C allele was more frequent than the T allele in the studied population as seen in Table 4.2, (*Khateeb S, 2025.*, n.d.). However, the deviation from Hardy–Weinberg equilibrium in the control group warrants cautious interpretation of these findings and highlights the need for larger, population-based studies to confirm the genetic distribution of TMPRSS6 variants in this region.

The high hepcidin levels in CHD patients in all TMPRSS6 genotypes (Figure 4.1) are probably due to the already known relationship between systemic inflammation and the triggering of hepcidin production. Moreover, the inter-genotypic variations depicted in Figure 4.5 indicate that there is a possibility of genetic modulation of hepcidin regulation. The outliers found in the group of CC

genotypes can be interpreted as evidence of substantial inter-individual variability or could also be defined by auxiliary factors, e.g. iron status or the age of a patient. Interestingly, the fact that the TT genotype is underrepresented in the control group can suggest a specific susceptibility of its genotype to hepcidin dysregulation in CHD. These results together favor the possibility of a connection between the TMPRSS6 A736V polymorphism and hepcidin homeostasis in this group of CHD patients.

Post-hoc analysis of the combined cohort, that is the control population and CHD population, revealed that the individuals with TT genotype had very high levels of serum hepcidin compared to those with the CC and CT genotypes. This observation implies that the TMPRSS6 A736V A736 V polymorphism may modulate the effect of hepcidin regulation on the genes based on the genotype of the carriers, where the attenuated suppression of hepcidin production in TT carriers. However, to further explain these relationships, a stratified post-hoc analysis in each group separately is necessary to reflect possible group-specific interactions.

Serum hepcidin levels differed significantly among the three TMPRSS6 A736V genotypes in the control group alone, with CC carriers showing lower hepcidin levels compared to both CT and TT carriers. No significant difference was observed between CT and TT carriers. These findings are consistent with the role of TMPRSS6 in the negative regulation of hepcidin expression, leading to genotype-dependent differences in hepcidin production. Specifically, carriers of the CC genotype may have more active TMPRSS6, resulting in reduced hepcidin synthesis, whereas the presence of the T allele appears to be associated with higher hepcidin levels. These results align with previous studies reporting elevated hepcidin in carriers of the T allele and support the functional impact of TMPRSS6 A736V on iron homeostasis (Pelusi et al., 2013a).

On the other hand, in the CHD cohort, the three genotypes had significant mutual differences, with a gradual increase in the hepcidin concentrations between

the CC and CT to TT. These data confirm the hypothesis of dose-dependent impact of the T allele, which seems to be enhanced in the situation of chronic disease and systemic inflammation. The increased levels of genotypic discrimination in CHD patients might be attributed to a possible synergistic effect between TMPRSS6 genetic variability and inflammatory triggers such as increased IL-6 levels which was shown to increase hepcidin expression. These results highlight how the disease status should be considered when evaluating the functional effects of the TMPRSS6 A736V polymorphism on hepcidin regulation with genetic effects appearing to be increased in inflammatory situations. Consequently, detecting these genetic predispositions can help to develop customized therapeutic approaches to regulate the activity of the hepcidin-ferroportin axis in order to manage anemia in CHD patients.

The distribution of underlying causes of end-stage renal disease observed in this study reflects epidemiological pattern in hemodialysis populations, where diabetes mellitus and hypertension remain the leading contributors to renal failure. The high prevalence of diabetes, either alone or in combination with hypertension, underscores the progressive impact of metabolic and vascular complications on renal function.

Hypertension-related causes further highlight the role of chronic cardiovascular stress in accelerating renal damage. Medical and post-surgical causes, including drug toxicity and complications following cardiac surgery, suggesting secondary factors to renal deterioration in this cohort. In contrast, hereditary and metabolic disorders such as primary oxalosis were relatively infrequent, consistent with their lower prevalence in the general population. The presence of cases with unknown etiology may reflect late referral, limited diagnostic data, or overlapping risk factors, which are common challenges in patients reaching end-stage renal disease.

5.2. Conclusion:

In conclusion, the present study demonstrates that chronic hemodialysis patients experience marked disturbances in iron metabolism, characterized by altered serum hepcidin levels, functional Iron deficiency, and anemia, reflecting the combined effects of impaired renal function and chronic inflammation. Our findings further reveal a significant association between the TMPRSS6 A736V polymorphism and hepcidin regulation, indicating a genotype-dependent effect that is more pronounced in patients undergoing chronic hemodialysis.

Moreover, the positive correlations between hepcidin and both ferritin and creatinine emphasize the central role of hepcidin as a key mediator linking iron stores, inflammation, and renal dysfunction. In contrast, the lack of significant associations with other hematological or iron indices highlights the multifactorial nature of hepcidin regulation in CHD, where genetic variability, iron status, inflammation, and kidney impairment interact in a complex manner.

Collectively, these findings underscore the importance of considering both genetic background and disease status when evaluating iron homeostasis in hemodialysis patients. Finally, further studies incorporating inflammatory markers such as Interleukin-6 are needed to confirm these observations and to support a more individualized approach to anemia management in chronic kidney disease.

5.3. Limitations

The study also has a number of limitations that include the geographical limitation that limits the participants that are available in Jenin city in the period of study. As a result, these findings cannot be generalized to other areas by logistical and travel limitations in the West Bank, which required a localized method of sampling. This local emphasis can also explain the deviation of Hardy Weinberg equilibrium in the control group-a finding that could be explained by a lack of sufficient sample size, stratification of populations or the lack of accuracy in genotyping. Moreover, financial and logistical constraints did not allow conducting additional laboratory tests, which would probably not reveal some subtleties of iron

status or inflammatory characterizations. Irrespective of these methodological limitations, this study gives initial but promising results on the contribution of the TMPRSS6 A736V variant to regulate iron homeostasis in CHD patients in Palestine.

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Appendices

Appendix 1. Institutional Review Board (IRB) of Arab American University

Arab American University
Institutional Review Board - Ramallah

الجامعة العربية الأمريكية
مجلس اخلاقيات البحث العلمي – رام الله

IRB Approval Letter

Study Title: "The Effect of TMPRSS6 A736V Polymorphism on Hepcidin Level and Iron Metabolism in Chronic Hemodialysis Patients in Jenin City-Palestine".

Submitted by: Hadeel Naser Abu Zinea

Date received: 4th September 2024

Date reviewed: 8th September 2024

Date approved: 10th September 2024

Your Study titled **"The Effect of TMPRSS6 A736V Polymorphism on Hepcidin Level and Iron Metabolism in Chronic Hemodialysis Patients in Jenin City-Palestine"** with the code number **"R-2024/A/142/N"** was reviewed by the Arab American University Institutional Review Board - Ramallah and it was approved on the 10th of September 2024.

Sajed Ghawadra, PhD
IRB-R Chairman
Arab American University of Palestine

الجامعة العربية الأمريكية - فلسطين
مجلس اخلاقيات البحث العلمي - رام الله
IRB-R
ARAB AMERICAN UNIVERSITY-PALESTINE
INSTITUTIONAL REVIEW BOARD - RAMALLAH

General Conditions:

1. Valid for 6 months from the date of approval.
2. It is important to inform the IRB-R with any modification of the approved study protocol.
3. The Bord appreciates a copy of the research when accomplished.

رام الله – فلسطين

Tel: 02-294-1999 **E-Email:** IRB-R@aaup.edu **Website:** www.aaup.edu

Appendix 2. Approval to allow the collection of patient samples and the results of their previous tests registered with the Palestinian Ministry of Health.

State of Palestine
Ministry of Health
Education in Health and Scientific
Research Unit

دولة فلسطين
وزارة الصحة
وحدة التعليم الصحي
والبحث العلمي

Ref.: .....
Date:.....

رقم: ٢٠٢٤/٤٤٨/١٦٤
تاريخ: ٢٠٢٤/٠٩/٠١

الأخ مدير عام الادارة العامة للمستشفيات المحترم،،،
عطوفة الوكيل المساعد للمهن الصحية المساندة المحترم،،،
الاخت ق. أ. مدير عام الادارة العامة لتكنولوجيا المعلومات المحترم،،،

تحية واحترام،،

الموضـــوع: تسهيل مهمة بحث

يرجى تسهيل مهمة الطالبة: هديل ناصر محمد ابو زينة – ماجستير علم الدم والمناعة– الجامعة العربية الامريكية، بعنوان:

تأثير الجين تي.ام.بي.ار.اس.اس.6 أ736ف على مستوى الهيبسيدين و الحديد لدى مرضى غسيل الكلى المزمن في مدينة جنين–فلسطين

حيث ستقوم الطالبة بجمع معلومات من خلال تعبئة استبانة من قبل المرضى بعد اخذ موافقتهم ومراجعة ملفاتهم، وجمع عينات دم من العينات التي تم سحبها او سحب عينة، وذلك في:

- مستشفى جنين

مع العلم ان مشرف الدراسة: د. فكري سمارة.
على ان يتم الالتزام بالمحافظة على اخلاقيات البحث العلمي وسرية المعلومات، وعدم التعرض للمعلومات التعريفية للمشاركين.
على ان يتم تزويد الوزارة بنسخة PDF من نتائج البحث، التعهد بعدم النشر لحين الحصول على موافقة وزارة الصحة.

مع الاحترام،،

د. عبدالله القواسمي
رئيس وحدة التعليم الصحي والبحث العلمي

نسخة: عميد كلية الدراسات العليا المحترم/ الجامعة العربية الامريكية

Telfax.:09-2333901 scientificresearch.dep@gmail.com تلفاكس: 09-2333901

Appendix 3. CHD Questionnaire – English format

CHD Questionnaire

A. Participant Information			
Name	_____	surname	_____
Age	_____	DOB:	_____/_____/_____
Weight:	_____ Kg	Height:	_____ Cm
Gender ☐ Female ☐ Male		Phone:	
Marital status ☐ single ☐ married ☐ divorced ☐ Widow ☐ skip			
Area ☐ urban ☐ Rural			
Locality (address)	_____	District	_____

B. Medical history				
Date of onset of dialysis:	_____/_____/____			
Duration of each dialysis session:	_____ hours			
Frequency of dialysis sessions per week:	Once	twice	thrice	Other (please specify): _____
Type of dialysis membrane used:	Synthetic	Biocompatible	I don't know	
Root cause of your chronic kidney disease:	Diabetes	Hypertension	Glomerulo nephritis	Other (please specify): _____
Have you undergone genetic testing for mutations related to iron metabolism?	Yes	No		
Have you experienced any side effects from dialysis?	Yes	No		
Have you been tested for HFE gene mutations	Yes	No		

(C282Y, H63D)?			
Are you currently taking erythropoiesis-stimulating agents (Epo)?	Yes (please specify frequency):_____		No
What is your known serum ferritin level?	_____ ng/ml		I don't know
What is your transferrin saturation (TS) percentage?	_____%		I don't know
Have you ever received intravenous (i.v.) iron supplementation?	Yes (please specify frequency): _____		No
What is your current hemoglobin level?	_____ g/dl		I don't know
Do you have any known inflammation markers (e.g., C-reactive protein levels)?	Yes	No	I don't knov
Has your serum hepcidin been measured?	Yes (please provide the result in nM): _____	No	I don't knov
Are you a smoker?	Yes		No

Appendix 4. Coronary Artery Disease Questionnaire – Arabic format

أ. معلومات المشارك			
اسم الشخص	_____	اسم العائلة	_____
العمر	_____	تاريخ الولادة	_____/_____/_____
الطول	_____ سم	الوزن	_____ كغم
الجنس	☐ أنثى ☐ ذكر	رقم الهاتف المحمول: _____	
الحالة الاجتماعية	☐ متزوج/ة ☐ أعزب/عزباء ☐ مطلق/ة ☐ أرمل/ة ☐ تخطى		
السكن	☐ مدينة ☐ قرية ☐ مخيم		
العنوان	_____	المنطقة	_____

ب. التاريخ الطبي

تاريخ بدء الغسيل الكلوي	_____/_____/_____
مدة كل جلسة غسيل	_____ساعات
عدد جلسات الغسيل الكلوي في الأسبوع	مرة / مرتان / 3 مرات / أخرى: _____
نوع غشاء الغسيل المستخدم	اصطناعي / متوافق حيويا / لا أعلم
السبب الرئيسي لأمراض الكلى المزمنة لديك	سكري / ارتفاع الضغط / التهاب كبيبات الكلى / أخرى: _____
هل خضعت لاختبارات جينية للكشف عن الطفرات المرتبطة بأيض الحديد؟	نعم / لا
هل واجهت أي آثار جانبية من الغسيل الكلوي؟	نعم / لا
هل تم اختبارك للطفرات الجينية HFE (C282Y، H63D)؟	نعم / لا
تتناول حالياً عوامل تحفيز إنتاج خلايا الدم الحمراء (Epo)؟	نعم (يرجى تحديد التكرار): _____ / لا
ما هو مستوى مخزون الحديد (الفيريتين) في مصل الدم لديك؟	_____ نانوغرام/مل / لا أعلم
ما هي نسبة تشبع الترانسفيرين TS لديك؟	_____% / لا أعلم
هل تلقيت من قبل مكملات الحديد عن طريق الوريد (i.v)؟	نعم (يرجى تحديد التكرار): _____ / لا

ما هو مستوى الهيموغلوبين الحالي لديك؟	_____غرام/ديسيلتر	لا أعلم
هل لديك أي علامات التهاب معروفة (مثل مستويات البروتين التفاعلي CRP؟)	نعم / لا / لا أعلم	
هل تم قياس مستوى الهيبسيدين في الدم لديك؟	نعم (يرجى تقديم النتيجة بالنانو مول): _____ / لا / لا أعلم	
هل أنت مدخن؟	نعم / لا	

Appendix 5. English informed consent

INFORMED CONSENT

AAUP-IRB-R Code No.: .....
AAUP-IRB-R Date: .....

I, ... *(Name of Participant /optional)* hereby agree to take part in the clinical research (clinical study/questionnaire study/drug trial) specified below:

Title of Study: The effect of TMPRSS6 A736V polymorphism on hepcidin level and iron metabolism in chronic hemodialysis patients in Jenin City-Palestine, Fulfillment of master degree, in Immunohematology, in AAUP.

(Name of program)

The nature and purpose of which has been explained to me by ....., and interpreted by ..... to the best of his/her ability in English.

I have been told about the nature of the research in terms of methodology, possible adverse effects and complications (as per Participant Information Sheet).
After knowing and understanding all the possible advantages and disadvantages of this research, I voluntarily consent of my own free will to participate in the clinical research specified above.
I understand that I can withdraw from this research at any time without assigning any reason whatsoever.

Date: ..... **Signature:**
.....

(Participant)

IN THE PRESENCE OF:

Name: .....

Designation: ..... **Signature:** .....

(Witness for Signature of Participant)

I confirm that I have explained to the participant the nature and purpose of the above-mentioned research.

Date: ..... **Signature:** .....

(Attending investigator)

Appendix 6 Arabic informed consent

نموذج الموافقة

AAUP-IRB-R Code No.: .....
AAUP-IRB-R Date: .....

أنا ..... (اسم المشارك / اختياري)

أوافق بموجبه على المشاركة في البحث المرجعي (الدراسة المرجعية / دراسة الانسجام / تجربة الأدوية) المحددة أدناه:

تأثير الجين تي.اد.بي.ار.اس.اس.6 أ736ف على مستوى الهيبسيدين و الحديد لدى مرضى غسيل الكلى المزمن في مدينة جنين فلسطين، لتحقيق درجة: الماجستير، في برنامج: علم الدم و المناعة.

تم شرح وتفسير طبيعة الدراسة وهدفها من طريق الباحث: .....

لقد تم إخباري عن طبيعة البحث من حيث المنهجية والآثار السلبية المحتملة والمضاعفات (حسب ورقة معلومات المشارك).

بعد معرفة وفهم جميع المزايا والعيوب المحتملة لهذا البحث، أوافق طواعية بمحض إرادتي على المشاركة في البحث المرجعي المحدد أعلاه.

أفهم أنه يمكنني الانسحاب من هذا البحث في أي وقت دون إبداء أي سبب على الإطلاق.

التاريخ: ..... إمضاء المشارك: .....

في حضور:-

اسم: .....

التسمية / اللقب: ..... إمضاء: .....

(شاهد على توقيع المشارك)

أؤكد أنني أوضحت للمشارك طبيعة وهدف البحث المذكور أعلاه.

تاريخ: ..... إمضاء: .....

(الباحث)

الملخص باللغة العربية

الخلفية: يُعدّ الهيبسيدين هرمونًا كبديًا رئيسيًا ينظم توازن الحديد في الجسم من خلال التحكم بامتصاصه وإطلاقه من البلاعم. ويتأثر تعبيره بحالة الحديد، والالتهاب، والعوامل الوراثية مثل جين TMPRSS6 لدى مرضى غسيل الكلى الدموي المزمن، ترتفع مستويات الهيبسيدين غالبًا نتيجة انخفاض الإطراح الكلوي والالتهاب المزمن، مما يؤدي إلى نقص حديد وظيفي وفقر دم وزيادة الحاجة إلى محفزات تكون الكريات الحمراء والحديد الوريدي. يؤثر تعدد الأشكال الجيني TMPRSS6 rs855791 (A736V)في تنظيم الهيبسيدين، حيث يرتبط الأليل Tبارتفاع مستوياته وانخفاض توفر الحديد.

الأهداف: :تقييم تأثير المتغير الجيني TMPRSS6 p.A736Vعلى مستويات الهيبسيدين في المصل ومعايير تكون الكريات الحمراء لدى مرضى غسيل الكلى الدموي المزمن الفلسطينيين، بهدف تحسين تدبير فقر الدم وضبط العلاج بالحديد والأدوية.

الطرق: تم جمع المعايير الدموية والكيميائية الحيوية والالتهابية من السجلات الطبية والاستبيانات. أُخذت عينات دم لقياس الهيبسيدين في المصل با ELISA، وتحديد النمط الجيني TMPRSS6 rs855791باستخدام تفاعل البوليميراز المتسلسل النوعي للأليلات.

النتائج: شملت الدراسة 50 مريض غسيل كلى دموي و50 شخصًا سليمًا. أظهر المرضى ارتفاعًا ملحوظًا في مستويات الهيبسيدين (الوسيط 525 مقابل 78.5 نانوغرام/مل، .(0.001 > pكان النمط الجيني CTالأكثر شيوعًا (66%)، يليه (23%) CCثم (11%) TT، مع زيادة تدريجية في الهيبسيدين من CCإلى CTثم .TTوُجد ارتباط إيجابي بين الهيبسيدين والفيريتين والكرياتينين، دون ارتباط مع الهيموغلوبين أو مؤشرات حجم الكريات أو الترانسفيرين. لم يكن للجنس أو التدخين تأثير، بينما كان السكري وارتفاع ضغط الدم من الأسباب الرئيسية للفشل الكلوي النهائي.

الخلاصة: ترتفع مستويات الهيبسيدين لدى مرضى غسيل الكلى الدموي المزمن اعتمادًا على النمط الجيني .TMPRSS6وتؤكد هذه النتائج أهمية أخذ التباين الوراثي لجين TMPRSS6بعين الاعتبار عند تدبير فقر الدم وتخصيص علاج الحديد ومحفزات تكون الكريات الحمراء لدى هؤلاء المرضى.

الكلمات المفتاحية:

غسيل الكلى الدموي المزمن، الهيبسيدين، TMPRSS6، A736V، استقلاب الحديد، فقر الدم، الفيريتين، التباين الوراثي.